EXPRESSION ANALYSIS OF ESTROGEN RESPONSIVENESS IN BREAST CANCER CELLS

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SHALONDA B. HALSEY

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ABSTRACT

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EXPRESSION ANALYSIS OF ESTROGEN RESPONSIVENESS IN BREAST CANCER CELLS

Advisor: Dr. K. Sean Kimbro

Dissertation dated July 2004

Estrogen responsiveness of breast cancers can be associated with the presence or absence of the estrogen receptors (ER). Identification and analysis of estrogen responsive genes is important for proper diagnosis and treatment of estrogen receptor alpha (ERα) negative breast cancer. The objective of this study was to identify differentially expressed genes associated with estrogen treatment of ERα-negative MDA-MB-231 breast cancer cells and ERα-positive 231α breast cancer cells. Total RNA isolated from estrogen treated and untreated cells was subjected to differential microarray examination using the Affymetrix U133A human GeneChip, which consisted of approximately 22,500 genes. Data analyses were performed by Microarray Suite (MAS) 5.0 and GeneSpring 6.0 software analysis systems. Over 10,000 genes and express sequence tags (ESTs) were differentially expressed in estrogen treated cells compared to nontreated cells. There were 529 genes with a 2-fold or higher increase in expression in estrogen treated MDA-MB-231 samples and 231 genes with a 2-fold or higher increase in

expression in estrogen treated 231α samples. Genes up-regulated in estrogen treated cells included BRCA1-associated protein 2 (BRAP2), ataxia telangiectasia mutated-Rad3 related (ATR), vascular endothelial growth factor (VEGF) and several ESTs. This study demonstrated that estrogen treatment altered the expression of a diverse group of genes in ERα-negative breast cancer cells, thereby indicating that those genes are activated through mechanisms independent of ERα. The estrogen responsiveness of ERα-negative cells has implications for improved therapeutics of all types of breast cancers.

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LIST OF ABBREVIATIONS

AF-1, -2	Activation Function 1,2
ATM	Ataxia Telangiectasia mutated
ATR	Ataxia Telangiectasia-related protein
BLAST	Basic Local Alignment Search Tool
BRAP2	BRCA1-associated protein 2
BRCA 1,2	Breast Cancer 1, 2
CDC2	Cell Division Cycle 2
cDNA	Complementary Deoxyribonucleic Acid
CE	Catechol Estrogen
CHK 1,2	Checkpoint Kinase 1, 2
DCIS	Ductal Carcinoma In Situ
E2	17 beta-Estradiol, Estrogen
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
ERE	Estrogen Responsive Element
ERK 1/2	Extracellular signal-Regulated Kinase
ESR2	Estrogen Receptor 2
EST	Expressed Sequence Tag
FRP1	FKBP12-rapamycin Associated Protein-Related protein

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPx	Glutathione Peroxidase
hERRa	Human Estrogen Receptor-Related alpha
IDC	Infiltrating Ductal Carcinoma
IGF2R	Insulin-like Growth Factor 2 Receptor
IGFB	Insulin-like Growth Factor Binding protein
ILC	Infiltrating Lobular Carcinoma
LB	Luria Broth
LCIS	Lobular Carcinoma In Situ
MAS 5.0	Microarray Suite 5.0
MBRL	Molecular Biology Research Laboratory
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Center of Biotechnology Information
NEO	Neomycin Resistant Gene
NLM	National Library Medicine
PBS	Phosphate Buffered Saline
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3-kinase
PR+	Progesterone Receptor positive
ROS	Reactive Oxygen Species

RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
STAT6	Signal Transducer and Activator of Transcription 6
TB	Terrific Broth
TCF7L1	Transcription Factor 7-like, T-cell specific
TGFIF2	Tumor Growth Factor beta-induced Factor 2
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

Breast cancer is one of the most common forms of cancer among women in the United States. It has been estimated that approximately 12.6 percent of American women will develop breast cancer during their lifetime. The onset of breast cancer is associated with the overexpression, and/or amplification of a number of genes including the ones encoding growth factors and growth factor receptors (Chrysogelos, S.A. et al. 1994). In the African-American population breast cancer appears to be aggressive, hormonally non-responsive and generally estrogen receptor alpha (ERα) negative (Elmore, J. et al. 1998; Joslyn, S.A. and West, M.M. 2000). Due to such observations, it is evident that significant disparities in mortality rates exist between African-American and Caucasian patients with breast cancer. The reason for such disparities is attributed to biological differences between the races (Elmore, J. et al. 1998). However, some social scientists suggest that the differences in socioeconomic status, limited access to health care, and differences in cancer treatment contribute to the disparities in survival among African-American women (Greenwald, H. et al. 1996; Simon, M. et al. 1997; Heck K. et al. 1997). Considering this disparity between African-American women and women in other populations with regard to ERα status, determining differential gene regulation due to estrogen could provide for more effective diagnosis and treatments.

In order to study the hormonal and molecular controls of growth and death in breast cancer cells, an expression profile of estrogen responsive genes in breast cancer should be established. The development of an expression profile is necessary because it can provide the opportunity to selectively target genes for disease intervention by appropriate drug development and gene therapies (Martin, K. et al. 2000). Previous studies have utilized DNA microarray analyses to identify differences in gene expression between estrogen treated and untreated ER-positive breast cancer cells, but there is little known literature pertaining to estrogen treated ER-negative breast cancer cells using global gene expression analysis. This study sought to determine the genetic profiles of breast cancer cells with regard to ER status.

1.1 Objective

This study attempted to determine non-ERα responses by:

a) Establishing gene expression profiles of ERα negative breast cancer cells following estrogen treatment.

b) Establishing gene expression profiles of ERα positive breast cancer cells following estrogen treatment.

c) Studying the expression of estrogen responsive genes in ERα-negative/positive breast cancer cell lines.

The goals of this study were to determine possible ERβ and non-ERα gene targets in ERα negative breast cancer cells, and ultimately provide further insight into the development of improved therapeutics for treating breast cancer of all etiologies.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

The breasts are composed of fatty, connective, and lymphatic tissue. There are lobules, which make up the mammary glands for milk production during late pregnancy and after childbirth. The lobules are arranged around ducts that transport milk to the nipples for nursing.

Breast cancer is a malignant tumor that results from uncontrolled cell growth in breast tissue. These cancerous cells replicate rapidly and may eventually spread to other parts of the body in a process known as metastasis. The most common types of breast cancer include ductal carcinoma in situ (DCIS), infiltrating ductal carcinoma (IDC), infiltrating lobular carcinoma (ILC) and lobula carcinoma in situ (LCIS). DCIS is noninvasive and curable in nearly all women. LCIS is not a "true" cancer but increases a woman's risk of developing cancer in the future. IDC and ILC are invasive tumors that invade the surrounding fatty tissue of the breast and can spread to other parts of the body.

Excluding cancers of the skin, breast cancer is the second most commonly diagnosed cancer among women. It is also the second leading cause of deaths due to cancer. It was estimated that 211,300 women in the United States would be diagnosed and 39,800 deaths would occur from breast cancer in 2003 (American Cancer Society).

Currently, there are over 2 million women living in the United States who have been treated for breast cancer.

The aggressive behavior that is displayed in breast tumors of African-American women is characterized by an increase in necrosis as well as tumors that are larger in size (Elmore, J. et al. 1998). Tumors from African-American patients have been reported to have a higher rate of estrogen receptor negativity, in addition to tumors that are more likely to have inflammatory, medullary and papillary histology (Joslyn, S. 2002). Investigations have taken place in order to gain insight into the factors that may be responsible for the aggressive tumors found in African-American women. A study of the estrogen receptors in tumors derived from African-American women indicated there were alterations in the ERα mRNA (Koduri, S. et al. 2000). Poola et al. (2002) presented data that illustrated that functionally active ER isoform profiles in breast tumors of African-American women were different from those in Caucasian women. Specifically, the tumors in African-American women were characterized by decreased levels of the protective ERβ isoform and elevated levels of the constitutively active ERα exon 5-deleted isoform.

It is not known exactly what causes breast cancer, but there are certain risk factors that are known to be linked to the disease. A number of factors, such as age, family history, a woman's age at birth of first child, early menarche and late menopause, are not modifiable. Alcohol consumption, use of postmenopausal hormones and obesity after menopause are other factors that are modifiable. Besides being female, age is a woman's single most important risk factor for developing breast cancer (Henderson, I.C. et al.

1995; American Cancer Society 2001). Currently, about 8 out of 10 breast cancers are found in women over age 50 (American Cancer Society 2003). Breast cancers that result from inherited mutations in breast cancer susceptibility genes, such as BRCA1 and BRCA2, account for approximately 5–10 percent of all cases (Hyde, J. et al. 1992; Burke, W. et al. 1997). At this time, there is no known strategy to eliminate all risk of breast cancer; however, a woman's best strategy is to minimize her known risk factors whenever possible.

When a person has been diagnosed with breast cancer, choosing the best form of treatment or management of the disease is important. After an examination of the medical circumstances and the patient's preferences, treatment may involve surgery, radiation therapy, chemotherapy, or hormone therapy (American Cancer Society 2003). Oftentimes, two or more of these methods are used in combination to effectively treat the disease.

2.2 Estrogen and Estrogen Receptors

There are many estrogens and estrogen-like molecules. 17β-estradiol is a steroid derived from the conversion of androgen in the gonads, brain and adipose tissue through the P-450 superfamily member, aromatase. Androstenedione and testosterone are the obligatory precursors of estrogens. The P450 aromatase monooxygenase enzyme complex catalyzes the conversion of androstenedione and testosterone into estrogens (Gruber, C. et al. 2002). Estrogen plays an essential role in the development of various tissues and in the maintenance of numerous physiological processes (Charpentier, A. et al. 2000). In paradox, it has been well documented that estrogen has a critical role in the

etiology and progression of human breast and gynecological cancers (Henderson, B. et al. 1982; Bernstein, L. et al. 1993; Pike, M. et al. 1993; McGonigle, K. et al. 1994; Adlercreutz, H. et al. 1994; Toniolo, P. et al. 1995).

Estrogen action is mediated through estrogen's ability to bind to the estrogen receptors (ER), α and β. The estrogen receptors are ligand-activated transcription factors that are members of the steroid receptor superfamily. In short, estrogens bind to the ER in a target tissue; the ER is then activated by changes in the three-dimensional shape of the estrogen-ER complex. This complex dimerizes and binds to specific sites in the promoter region of estrogen-responsive genes. Transcription of target genes is initiated by coactivators and other regulatory proteins that bind to the external surface of the immobilized ER complex (Jensen, E. et al. 1962; Gorski, J. et al. 1968; Jensen, E. et al. 1982; Kuiper, G. et al. 1996; Mosselman, S. et al. 1996; Bentrem, D. et al. 2003). The transcription complex either binds directly to DNA at an estrogen responsive element (ERE) or is tethered independently at AP-1 or SP-1 sites (Steinmetz, A. et al. 2001; Nilsson, S. et al. 2001; Bentrem, D. et al. 2003). Figure 1 represents the classic estrogen receptor signaling pathway. A new role of estrogens as mediators of rapid non-genomic effects has recently been identified, in addition to the classical genomic regulation of target gene expression. These rapid effects include the stimulation of nitric oxide release in vascular cells and the activation of Ras and Raf-1 kinase, resulting in the activation of ERK1/2 and the phosphorylation of transcription factors in both non-myocytes and cardiac myocytes (Chen, Z. et al. 1999; Kim, H. et al. 1999; Pratt, M. et al. 1998; Endoh,

H. et al. 1997; Russell, K. et al. 2000; Singer, C. et al. 1999; Watters, J. et al. 1997; Improta-Brears, T. et al. 1999; Nuedling, S. et al. 2000; de Sager, T. et al. 2001). These non-nuclear estrogen signaling events are thought to be mediated by ERs at the cell surface/plasma membrane (Ramirez, V and Zheng, J. 1996; Wade, C. et al. 2001; Santen, R. et al. 2002; Stoica, G. et al. 2003).

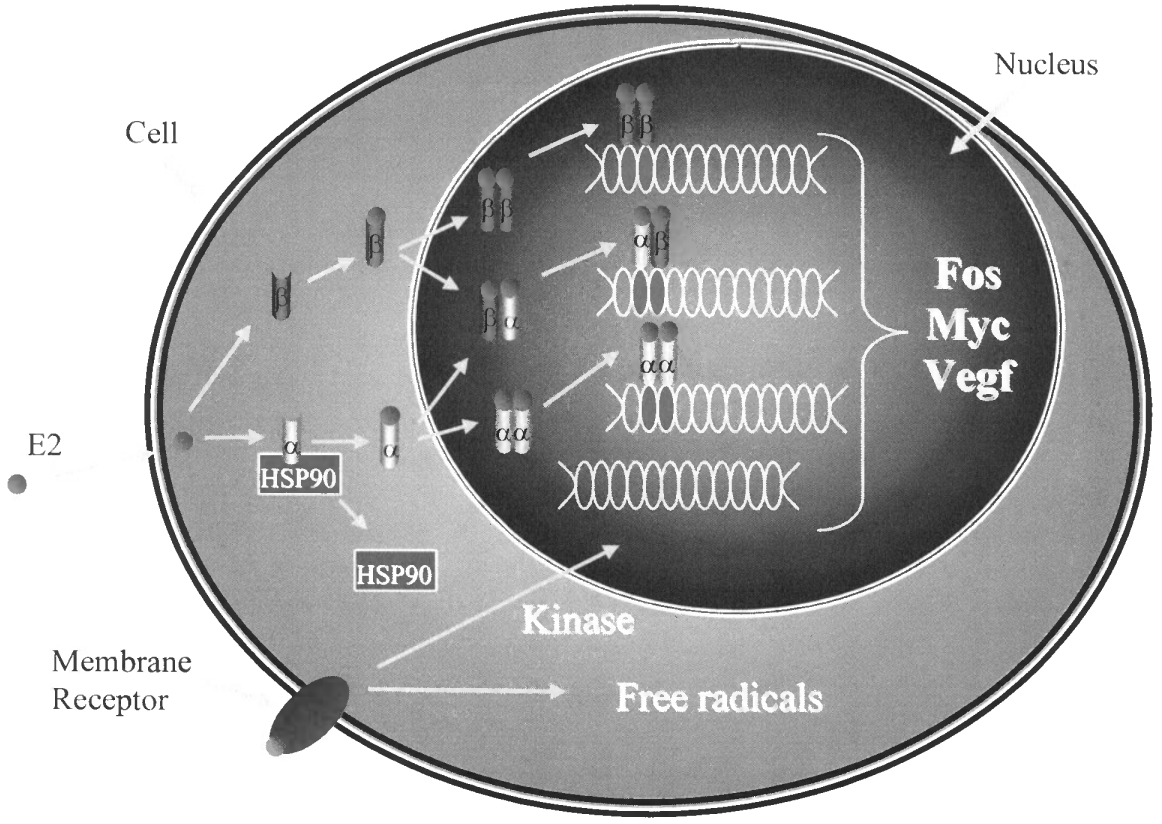


Figure 1. Estrogen signaling pathway. Estrogen (E2) binds to the estrogen receptors α or β. The estrogen-estrogen receptor complex can form as heterodimers or homodimers with the estrogen receptors. The complex then binds to DNA at estrogen responsive elements, initiating transcription.

The identification of a second ER added a new dimension to the complexity of estrogen signaling. ERβ was identified in Gustafusson's laboratory in 1996 (Kuiper, G.

et al. 1996). Since the initial identification, five ERβ isoforms have been cloned and characterized (Tong, D. et al. 2002). ERβ has a structure and function similar to ERα, but its prognostic factors remain to be well established in breast carcinoma (Iwao, K. et al. 2000). ERβ has been shown to bind to estrogen with an affinity resembling that of ERα and activates expression of receptor genes containing estrogen response elements in an estrogen-dependent manner (Pace, P. et al. 1997; Dotzlaw, H. et al. 1996). Although ERβ has significant levels of expression in the central nervous system, cardiovascular and immune systems, urogenital tract, bone, kidney and lung, it is also expressed in breast cancer and is regulated by estradiol (Gustafusson, J. 2000; Vladusic, E. et al. 2000).

Recent studies indicated that the loss of ERβ expression could be one of the events leading to the development of breast cancer, due to it regulating proliferation and invasion of breast cancer cells (Lazennec, G. et al. 2001). Most breast tumors tend to express ERα, but it has been seen in combination with ERβ (Speirs, V. et al. 1999a; Jarvinen, T. et al. 2000). Dimerization of ERβ and ERα indicate that the DNA binding domain of ERα is sufficient to dimerize with ERβ (Pace, P. et al. 1997). The mere existence of ERβ and its dimerization with ERα add greater levels of complexity to transcription and activation in response to estrogens. The coexpression of the two ER subtypes is associated with tumors with a poor prognosis, which may explain the failure of antiestrogen therapy in some breast cancer patients (Speirs, V. et al. 1999b).

ERα and ERβ also share homology in their central DNA-binding and carboxyl-terminal ligand binding domains, but there is very little similarity between the two

subtypes in the amino-terminal domain (Delaunay, F. et al. 2000). It is suggested that the amino-terminal region plays an important role in the control of the transcriptional activity exhibited by ERα and ERβ (Paige, L. et al. 1999). The transcriptional activity of ERα has two different transcription activation functions (AF), AF-1 and AF-2, located in the amino-terminal region and in the carboxyl-terminal region of the ligand-binding domain, respectively (Tora, L. et al.1989; Barkhem, T. et al. 1998). The function of the AF-1 site is hormone-independent, while the AF-2 site requires the presence of hormone for activation (Tora, L. et al.1989). Even though ERβ shares this similarity, the amino-terminal domain of ERβ is 80 amino acids shorter and illustrates no sequence homology with that of ERα (Delaunay, F. et al. 2000; Ogawa, S. et al 1998). To date it is known that ERα and ERβ have distinct cellular distributions, regulate separate sets of genes and can oppose each other's actions on some genes. It is also known that ERβ is widely expressed in both normal and malignant breast tissue and that there are proliferating cells in the breast which express ERβ. However, it is still unclear whether ERβ could serve as a good prognostic indicator, along with or independent of ERα, of breast cancer development.

Estrogen is also known to contribute to the development and progression of breast cancer due to prolonged stimulation of breast ductal epithelium (Osborne, C. et al. 2000). The mitogenic effect of estrogen that results due to its interaction with ER allows for treatment regimens that include antiestrogens, synthetic compounds which oppose the aetions of estrogens (Gottardis, M. et al. 1988; Sunderland, M. et al. 1991). This type of treatment is known as hormonal therapy; it uses hormones to prevent the growth,

spread or recurrence of breast cancer. Research has indicated that hormonal therapy can extend the lifespan of a person diagnosed with breast cancer with cancer cells that depend on hormones for proliferation. One of the most common drugs used for hormonal therapy is tamoxifen. Tamoxifen is used to treat patients with advanced stage breast cancer as well as patients with early stage breast cancer. Use of tamoxifen is determined by the patient's ER status. Two-thirds of all breast cancer patients with ER-positive tumors will benefit from tamoxifen treatment, but most patients will eventually relapse (Speirs, V. et al. 199b). The tamoxifen resistant development is thought to occur due to an increased expression of ERβ (Garcia, M. et al. 1992). Further research is needed to develop synthetic estrogen modulators that will combat breast tumors that are ERα-negative and ERα-positive.

2.3 Microarray Technology

With the human genome sequencing project as well as the advent of microarray technology, it is now possible to investigate the complexities of ER-mediated gene transcription on a more global scale, rather than one estrogen-responsive target gene at a time. DNA microarrays are used to simultaneously measure the expression levels of thousands of genes. New approaches based on microarray technology have been used to characterize distinctive gene expression patterns in human mammary epithelial cells and breast cancer (Perou, C. et al. 1999; Soulez, M. and Parker, M. 2001). This approach has also been used to monitor gene expression during breast cancer progression and to characterize breast tumors (Sgroi, D. et al. 1999; Nacht, M. et al. 1999; Bertucci, F. et al. 2000; Perou, C. et al. 2000; Soulez, M. and Parker, M. 2001).

The effects of estradiol on increased proliferation and tumorigenesis in breast cancer cells have been well documented, and several recent studies using microarray techniques have begun to document the gene expression profiles in breast cancer. The focus of most of these studies has been on identifying genes overexpressed in breast cancer, patterns of gene expression associated with clinical outcome or prognosis, responses to chemotherapy or drug resistance, tumor aggressiveness, and classification of primary tumors (Jiang, Y. et al. 2002; van de Vijver, M. et al. 2002; van't Veer, L. et al. 2002; Sotiriou, C. et al. 2002; Zajchowski, D. et al. 2001; Perou, C. et al. 2000; Sorlie, T. et al. 2001; Hedenfalk, I. et al. 2002). The role of ER expression has also been addressed in several studies in which distinct gene expression patterns associated with ER status have been identified (West, M. et al. 2001; Dressman, M. et al. 2001; Gruvberger, S. et al. 2001; Frasor, J. et al. 2003). Even with these recent studies, the exact role of estrogen-mediated gene regulation in ER-positive as well as ER-negative breast cancer and the manner by which these changes in gene expression affect breast cancer proliferation and progression is still unclear. Few microarray investigations have examined the role of estrogens in the regulation of gene expression, and the studies that have been done in breast cancer cells have been on a relatively limited scale, with few genes examined over a limited time course of hormone treatment (Soulez, M. and Parker, M. 2001; Omoto, Y. and Hayashi, S. 2002; Levenson, A. et al. 2002; Inoue, A. et al. 2002; Lobenhofer, E. et al. 2002).

Current investigations by Frasor et al. (2003) have highlighted the diverse gene networks and metabolic and cell regulatory pathways through which estradiol operates to

achieve its widespread effects on breast cancer cells. Cunliffe et al. (2003) performed comparative analysis using gene expression patterns from *in vitro* and *in vivo* studies which revealed a significant association between specific gene clusters and differentiation in hormone receptor status and disease progression in tumor samples. Though these findings are very significant, they neglect to address or expound on gene profiles representing ER-negative breast cancer.

Genes associated with ER-negative status are of particular interest because they may reveal the biological causes of the distinct behavior of these tumors (Pusztai, L. et al. 2003). ER-negative tumors usually develop more often in premenopausal women, may recur sooner, and may spread more commonly to the lung, liver and the central nervous system (Clark, M. et al. 1996). Therefore, establishing and elucidating the mechanisms that cause regulation of ER-negative genes could provide potential targets for drug development.

The question still remains how estrogen activation is mediated through ERβ or non-genomic mechanisms in ERα-negative breast cancer cells. Therefore, it is important to study the effects of estrogen on gene expression in cells that are ERα-negative. The main objective of this research was to determine genetic profiles of breast cancer cells with regard to ER status. Expression profiles were established for ERα-negative and positive breast cancers following estrogen treatment. Estrogen responsive genes were identified and analyzed by Microarray Suite (MAS) 5.0 (Affymetrix, Santa Clara, CA) and GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA). Results were confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR).

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Culture

Human breast cancer cell lines, MDA-MB-231 and MCF-7, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The stable transfectants used in this study were constructed from the ERα-negative MDA-MB-231 cells and will be described later. All cell lines were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% newborn calf serum, 10,000 U/ml penicillin and 10 mg streptomycin, fungizone and 5 mg/ml gentamycin sulfate and incubated at 37°C in 5% CO_2. Prior to estrogen exposure, confluent cells were cultured for 24 hours in phenol-red free RPMI 1640 medium containing 10% charcoal stripped newborn calf serum supplemented with the previously mentioned antibiotics.

3.2 Plasmids and Transformations

The full-length human estrogen receptor-alpha (hERα) cDNA was provided in a pcDNA3 expression vector (pcDNA3hERα) as a generous gift from Dr. Kenneth Korach (NIEHS, National Institutes of Health, Bethesda, MD). In addition, pcDNA3 expression vector was purchased from Invitrogen (Carlsbad, CA) to use as a negative control for pcDNA3hERα. Transformations were performed by electroporation (Electro Cell Manipulator®, ECM 600, BTX, San Diego, CA) using XL-1 Blue and JM109

electrocompetent cells (Stragene, La Jolla, CA and Promega, Madison, WI), respectively. The vectors were diluted 1:10 and 1 µl was added to 400 µl of cells, then incubated on ice for 10 minutes. The DNA-cells mixtures were pulsed according to the electroporation conditions listed below.

Mode:	T	High Voltage/Resistance
Capacitance:	C	50µF
Resistance:	R	R4 (72ohms)
Charging Voltage:	S	150V

Nine hundred sixty microliters of Luria Broth (LB, Miler's Modification, Sigma, St. Louis, MO) was added immediately after pulse and then transferred to microcentrifuge tubes for incubation at 37°C for 30 minutes with agitation. The cells were then centrifuged at 2,000 rpm for 30 minutes. Media were removed and the pellet, though not visible, was resuspended in 200 µl of LB and spread on LB agar plates containing 100-150 µg/ml of ampicillin. The plates were incubated at 37°C overnight. Several colonies from the LB agar plates were grown in 250 ml of Terrific Broth (TB; tryptone, yeast extract, K_2HPO_4, KH_2PO_4) containing 150 µg/ml of ampicillin and incubated in a shaker at 180-200 rpm at 37°C overnight.

3.2.1 Plasmid DNA Isolation

The transformed bacteria cells were harvested by centrifugation at 6,000 rpm for 15 minutes. The supernatant was discarded and the pellet was placed on ice. Each plasmid was isolated and purified utilizing the QIAGEN Plasmid Maxi Prep System (Valencia, CA). The QIAGEN plasmid purification system involves treating the cells

with an alkaline cell lysis buffer to break open the cells followed by filtration on an anion-exchange filter. This anion-exchange filtration system contained resin, a positively charged molecule, that bound strongly to negatively charged double-stranded plasmid DNA while other weakly bound impurities, such as RNA and proteins, were washed away leaving pure DNA. The DNA concentrations were determined by absorbance at 260/280 nm ultraviolet light using a Beckman DU 650 spectrophotometer.

3.2.2 Restriction Enzyme Digestion

Restriction enzymes or endonucleases are bacterial enzymes that cleave both DNA strands at specific 4- to 8-bp nucleic acid sequences called restriction sites. The enzyme used in the digestion of pcDNA3hERα construct was EcoRI (Promega, Madison, WI). EcoRI enzyme is isolated from *Escherichia coli* and generates fragments with single-stranded regions or "sticky ends." The restriction enzyme digestion reaction was composed of 0.5-1 μg of plasmid DNA, 10X restriction enzyme buffer (Multi-core buffer; 25 mM Tris Acetate, pH 7.5, 100 mM potassium acetate, 10 mM Mg Acetate, 1 mM DTT), EcoRI (10 U/ml) and distilled, deionized water. The reaction was incubated in a 37°C water bath overnight. A 1% agarose (Fisher Scientific, Pittsburg, PA) gel made with 1X Tris Borate EDTA (TBE; tris base, boric acid, EDTA, dH_20) buffer was used in the electrophoresis of the restriction enzyme digestion.

3.2.3 Sequence Analysis

In addition to restriction enzyme analysis, sequence analysis was performed to verify the positive clone. The sequence analysis was performed utilizing the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City,

CA). This kit utilized the deoxynucleotide sequencing method in which modified DNA bases were incorporated into the DNA strand to terminate the extension of the strand by DNA polymerase. The dideoxynucleotides were labeled by the manufacturer with fluorescent dyes, and, through a series of reactions, the different terminator dyes fluorescences were read by the ABI Prism 377 DNA Sequencer. The sequencing reaction included: 200-500 ng of DNA, 0.5 μg primer (ERα 368 or 710) and 8 μl of the Terminator Ready Reaction Mix in a final volume of 20 μl. The Terminator Ready Reaction Mix contained dideoxynucleotides with the 6-carboxyfluorescein (6-FAM) linked to a dichlororhodamine (dRhodamine) dye and AmpliTaq DNA Polymerase, FS, a derivative of *Thermus Aquaticus* DNA that was modified to increase peak intensity of fluorescence.

The sequencing reaction was performed in a DNA thermal cycler, GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA) under the following PCR conditions; 96°C for 30 seconds followed by 25 cycles of denaturing at 96°C for 10 seconds, annealing at 50°C for 10 seconds, extension at 60°C for 4 minutes. The reaction was analyzed on an ABI Prism 377 Sequencer at the Molecular Biology Research Laboratory (MBRL) at Clark Atlanta University. Verification of the sequence was done by comparing with known sequences in the National Center of Biotechnology Information (NCBI) of the National Library of Medicine (NLM) database using the Basic Local Alignment Search Tool (BLAST).

3.2.4 Stable Transfection of ERα

The MDA-MB-231 breast cancer cell line was stably transfected with the ERα expression plasmid (pcDNA3hERα) and the control plasmid (pcDNA3) by electroporation (Electro Cell Manipulator®, ECM 600, BTX, San Diego, CA). Cells ($9.1x10^5$ cells/ml) were centrifuged at 1,000 rpm for 10 minutes to pellet and the pellet was resuspended in 10 ml of HeBS (20 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO4, 6 mM dextrose). Four hundred microliters of cells and 20 μg of DNA were added to a BTX disposable cuvette with a 2 mm chamber gap (BTX/Gentronics, San Diego, CA). The mixture was pulsed according to the electroporation conditions listed below.

Mode:	T	500 V Capacitance & Resistance Low Voltage
Capacitance:	C	1000 mF
Resistance:	R	R4 (72 ohms)
Changing Voltage:	S	150 V

After pulse, cells were incubated at room temperature for 5 minutes, and then transferred to a cell culture dish containing fresh growth media. The cells were grown for 48-72 hours before selection media containing 400 μg/ml Geneticin® (G-418 Sulfate, Invitrogen, Carlsbad, CA) was added. Geneticin® is an aminoglycoside selective agent related to gentamycin that blocks protein synthesis in mammalian cells by interfering with ribosomal function. Cells require the expression of the neomycin phosphotransferase gene that confers resistance to the antibiotic, Geneticin®. The stable transfectants continued to grow in selective media for 5 days. Selection continued for 5

more days in 600 μg/ml of Geneticin®. The remaining cell colonies were transferred using plastic cloning rings to 96-well plates (Fisher, Suwanee, GA) in the presence of non-selective media and grown until 90-100% confluency. This process continued until cell density was optimal for maintenance in tissue culture flasks.

3.3 Treatment of Cells with Estrogen

Seventeen (17) β-estradiol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of E2 was made in ethanol at 20 mg/ml and was stored at 2-8°C. A working concentration of $1x10^{-9}$ M was made directly in the tissue culture medium before treatment.

Cells were seeded in 150 mm cell culture dishes (Corning, Fisher, Suwanee, GA) in sets of 2 or 3 dishes per cell line for each treatment. When cells became 90-100% confluent, phenol-red free RPMI 1640 media containing 10% charcoal stripped newborn calf serum supplemented with the previously described antibiotics were added for 24 hours. The cells were rinsed twice with PBS, and fresh phenol-red free media was added containing $1x10^{-9}$ M of E2. The estrogen remained on the cells for 2 and 12 hours with the respective untreated controls. These time points were used to identify early and intermediate/late expressed genes that have not been identified as estrogen regulated genes.

3.4 Isolation of Total RNA

Total RNA was isolated from the cell lines using TRIZOL Reagent (Invitrogen, Carlsbad, CA) or QIAGEN RNeasy Kit (Valencia, CA). The TRIZOL reagent is a

monophasic solution of phenol and guanidine isothiocyanate. During sample lysis, the reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. The addition of chloroform is needed for purification and phase separation of the solution.

The RNeasy principle combines the selective binding properties of a silica-gel-based membrane with the speed of spin technology. A specialized high-salt buffer system allows up to 1 mg of RNA longer than 200 bases to absorb to the RNeasy silica-gel membrane. In short, samples are lysed and homogenized in the presence of a highly denaturing buffer containing guanidine isothiocyanate, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is added to the column where total RNA binds and contaminants are efficiently washed away. High-quality RNA is eluted in RNase-free water and ready for use.

After RNA isolation was completed, samples were resolved by electrophoresis on a 1% agarose gel to determine stability. Concentrations were determined by spectrophotometry at absorbance 260/280 nm.

3.5 Isolation of DNA

The tissue culture cells were rinsed with 1X PBS twice. Excess liquid was aspirated off the plate and 1 ml/10^8 cells of DNA digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8, 25 mM EDTA, pH 8, 0.5% Triton-X 100 or 0.5% SDS, 0.1 mg/ml proteinase K) was added. The cells were scraped into tubes appropriate for the volume and samples were incubated in a 50°C water bath for 1hr to overnight. A

phenol/chloroform extraction was performed by adding an equal volume of phenol saturated with Tris-Cl (pH 8.0) to the samples. The samples were mixed gently by inversion and centrifuged for 15-30 minutes at room temperature. The supernatant was transferred to another tube and an equal volume of chloroform was added. The samples were mixed by inversion and the phases were separated as previously described. DNA was precipitated by the addition of 0.6 volumes of 3 M sodium acetate and 2 volumes of 100% ethanol at –20°C for 30 minutes. After centrifugation, the pellet was air-dried for 5-10 minutes and resuspended in the appropriate volume of dH_2O. Concentrations were determined by UV spectrophotometry at absorbance 260 nm.

3.6 GeneChip Microarrays

The HG-U133A Array was used to measure the expression levels of a vast majority of genes. This array is a part of the HG-U133 set from Affymetrix, Inc. (Santa Clara, CA) that includes approximately 22,500 human genes. The total RNA was shipped to Medical College of Georgia (Augusta, GA) for processing. RNA was converted to cRNA and hybridized to the HG-U133A GeneChip according to standard Affymetrix protocol. Briefly, RNA was first converted to double-stranded cDNA using an oligo-dT primer with a T7 RNA polymerase site on the 5' end. It was then used directly in an in vitro transcription reaction in the presence of biotinylated nucleotides following manufacturer protocol (Enzo Bioarray High Yield RNA Transcript Kit, Farmingdale, NY). Labeled antisense RNA was then fragmented to improve hybridization kinetics, and hybridized to the high-density microarray. The chips were

washed, stained with phycoerythrin-streptavidin, and scanned according to the manufacturer instructions.

Initial analysis was performed using Microarray Suite 5.0 (MAS) software (Affymetrix). Average intensities for each GeneChip were globally scaled to a target intensity of 150. For each transcript, MAS 5.0 applied one-sided Wilcoxon signed rank test to each probe set on the experimental (2hr and 12hr) chip and compared it to its corresponding probe set on the baseline (control) chip. MAS 5.0 determined those transcripts that were differentially expressed based on user-defined limits. Further analysis was performed using GeneSpring 6.0 software (Silicon Genetics, Redwood City, CA) to obtain fold-change and p-values for each gene at each time point relative to untreated control.

Microarray data were generated from three independent microarray experiments performed for each timepoint per cell line. In order to aid in the selection of genes, specific criteria were generated. Fold induction was the first criterion; a cut-off of 2-fold increase or decrease was defined for selecting genes of interest. Only those genes that had similar expression levels in at least two independent experiments and showed at least a 2-fold increase or decrease in expression were selected as E2 regulated candidate genes for further analysis. The stringency was increased to 5-fold in order to select genes with the highest fold change. Secondly, genes were screened based on the raw intensity of each gene's signal. When microarray chips were scanned, each transcript on the array emitted a signal and the intensity was measured. The cut-off intensity was 150 units.

Genes were then prioritized according to those that were unknown or had an inferred function, transcription factors, receptors, breast cancer related and enzymes.

3.7 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

RNA was reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA) with random hexamer or oligo dT primers. Primers were designed using OLIGO® Primer Analysis Software version 4/5 (NBI, Plymouth, MN) for a select number of differentially expressed genes. Selection was based on the fold induction of genes identified by MAS 5.0. A list of primers is shown in Table 1. The cDNA was amplified in a 20 μl reaction mixture. The PCR conditions were 95°C for 3 min followed by 35 cycles of denaturing at 95°C for 30 sec, annealing at 58°-60°C for 45 sec, extension at 72°C for 2 min and a final extension at 72°C for 5 min. PCR products were analyzed on 1-2% agarose gel and imaged on a BioRad ChemiDoc System (Hercules, CA). For breast tissue samples, RNA was isolated using RNeasy kit (Qiagen, Valencia, CA). Synthesis of cDNA was performed using GeneAmp RNA PCR kit (Perkin Elmer, Boston, MA) and amplified by PCR.

Table 1. List of PCR primers

PCR PRIMERS
ERα 368 5' -GGG GAG GGC AGG GGT GAA GTG
ERα 710 5' -GGA GCG CCA GAC GAG ACC AAT CAT
GAPDH (forward) 5' -ACA CAT GCC ATC ACT GCC
GAPDH (reverse) 5' -GCC TGC TTC ACC ACC TTC TTG
ERβ (forward) 5' -TAG TGG TCC ATC GCC AGT TAT
ERβ (reverse) 5' -GGG AGC CAC ACT TCA CCA T
ERα (forward) 5' -AAT TCA GAT AAT CGA CGC CAG
ERα (reverse) 5' -GTG TTT CAA CAT TCT CCC TCC TC
NEO 634 5' -GTG TTC CGG TGT CAG CGC CA
NEO 1169 5' -GTC CTG ATA GCG GTC CGC CA
ESR2 466 5' -CAA CTG CAG TCA ATC CAT CTT ACC C
ESR2 663 5' - AAG GAG AAA GGT GCC CAG GTG
ERα 817 5' -AAT TCA GAT AAT CGA CGC CAG GGT G
ERα 1137 5' -GTG TTT CAA CAT TCT CCC TCC TCT T
ATR 3722 5' -CTT GTC TGG GCT CCC TTC TCA GTC
ATR 4170 5' -CCC CCA ATT CCC CTA AAC ATT CC

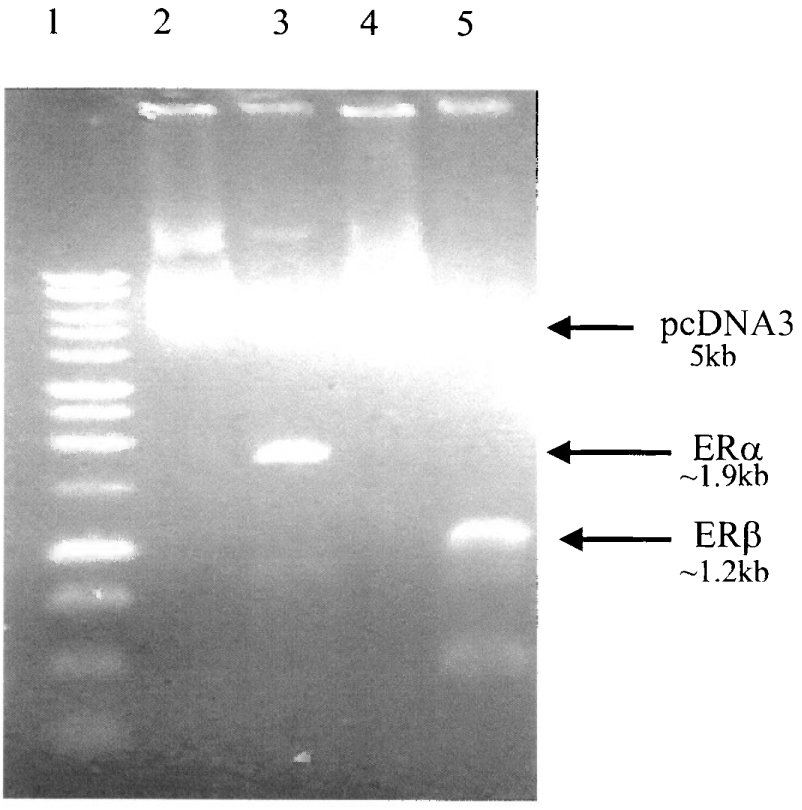
CHAPTER 4

RESULTS

4.1 Creation of Stable Transfectant Cell Lines

Stable transfectant cell lines, MDA-MB-231hERα (231α) and MDA-MB-231Neo6 (Neo 6), were created by electroporation of MDA-MB-231 breast cancer cells with ERα and an empty pcDNA3 vector, respectively. Prior to transfection, ERα plasmid DNA was isolated from transformed *E. coli* cells and restricted with EcoRI (Figure 2). The digestion reaction confirmed the presence of the ERα cDNA in the pcDNA3 vector. Further verification was performed by sequence analysis.

The pcDNA3 plasmid DNA isolated from transformed bacteria cells was analyzed by electrophoresis on a 0.7% agarose gel (Figure 3); therefore, confirming that the empty vector was incorporated. After a successful transfection and selection process, the cells were maintained in culture under normal conditions for several weeks prior to experimental use. A RT-PCR screening for the presence of the neomycin resistant gene (NEO) was performed to ensure the transfected cell line did contain the pcDNA3 construct. The primers that were used for this experiment are shown in Table 1. The results from the RT-PCR screening with NEO specific primers are shown in Figure 4. The NEO primers produced a band at a size of 550 bp on a 2% agarose gel. Samples from the MDA-MB-231 and 231α cell lines were also used. The MDA-MB-231 sample



Lane 1- 1kb Marker

Lane 2- ERα plasmid DNA

Lane 3- ERα plasmid DNA cut w/ EcoRI

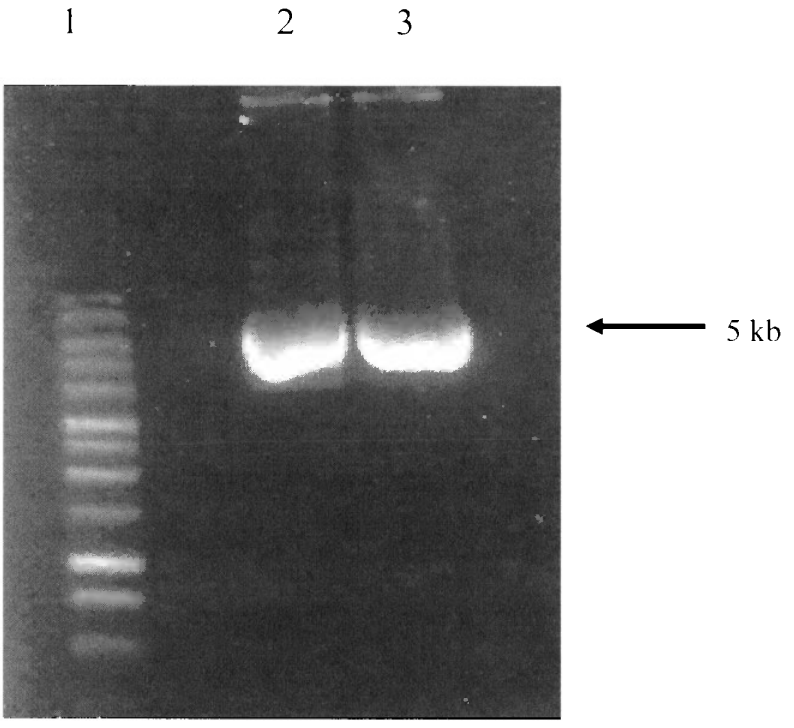
Lane 4- ERβ plasmid DNA

Lane 5- ERβ plasmid DNA cut w/ EcoRI

Figure 2. EcoRI digestion of ERα plasmid DNA. Plasmid DNA was isolated and purified from transformed *E.coli* cells. The DNA was utilized in a restriction digest reaction with EcoRI restriction enzyme to detect the presence of ERα cDNA in the vector. ERβ plasmid DNA was used to compare the sizes of the cDNA.

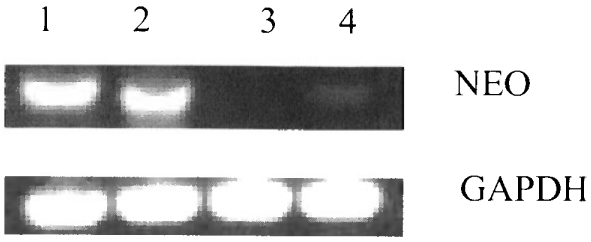
was negative for the NEO screening and the 231α sample was positive. Primers for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to confirm the success of the RT reaction and to verify that approximately equal amounts of

cDNA template was added to each reaction. GAPDH produced a band at an approximate size of 200 bp. The establishment of these cell lines facilitates the uniform analysis of ERα activity in an environment that is endogenously naive of ERα.



Lane 1- 1kb Marker
Lane 2 & 3- pcDNA3 plasmid DNA

Figure 3. pcDNA3 plasmid DNA. Purified plasmid DNA was analyzed by electrophoresis to confirm the presence of the empty pcDNA3 vector in the transformed *E. coli* cells.



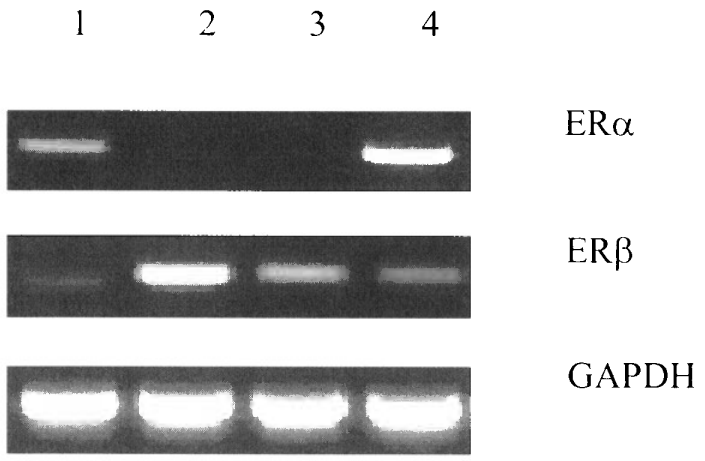
Lane 1 – 231α
Lane 2 – Neo 6
Lane 3 – MDA-MB-231
Lane 4 – MCF-7

Figure 4. RT-PCR screening for neomycin (NEO) resistant gene. RT-PCR was used to detect the expression of NEO in the transfected cell lines, 231α and Neo 6. The neomycin resistant gene is incorporated into the vector for selection of stable transfectant cell lines. GAPDH was used as an internal control to verify approximately equal cDNA templates in all samples.

4.2 ER Status of Breast Cancer Cell Lines

Previous studies have identified MCF-7 breast cancer cells as ER positive and MDA-MB-231 breast cancer cells as ER negative. In order to confirm these findings in our batch of cell lines, RT-PCR analysis was performed using ERα and ERβ primers. The stable transfectants, 231α and Neo 6, were also screened for the presence and/or absence of ERα and ERβ. Figure 5 shows the results from the RT-PCR screening for ER status. The ERα primers (817/1137) produced a band at a size of 320 bp on a 1.5% agarose gel in MCF-7 and 231α breast cancer cells. The ERβ primers (ESR2, 466/663) produced a band at a size of 197 bp on a 1.5% agarose gel in MDA-MB-231, Neo 6 and 231α cells. This set of ERβ primers produced a faint band in MCF-7 cells during this

particular experiment, but a broader band has been produced in previous experiments and with other ERβ primers. The faint expression of ERβ seen in Neo 6 and 231α cells could be the result of transfection. GAPDH produced a band at an approximate size of 200 bp in all sample.



Lane 1 - MCF-7
Lane 2 - MDA-MB-231
Lane 3 - Neo 6
Lane 4 - 231α

Figure 5. RT-PCR screening for ER status. Total RNA was extracted from each cell line and used in RT-PCR analysis to detect the presence of ERα and/or ERβ. GAPDH was used as an internal control to confirm approximately equal cDNA templates in all samples.

4.3 Effects of Estrogen on Cell Proliferation

Figure 6 shows the growth data of MDA-MB-231 cells after treatment for 96 hrs with 17β-estradiol. During the first 48 hrs, the control and E2 treated cultures showed no differences in growth. There was a 5.8% and 22% difference in growth at 72 hrs and 96

hrs, respectively, between E2 and control (at 96 hrs, P= 0.04). As shown in Figure 7, E2 and control treatment of 231α cultures showed no change in the rate of growth from 0-48 hrs. However, at 96 hrs, E2 stimulated growth 18.5% higher than control (P= 0.06). Figure 8 illustrates the effect of E2 treatment on proliferation of Neo 6 cells. Overall, the growth of the cells was inhibited in the presence of E2 and in the untreated control cells. This could be due to the mere presence of the empty vector in these cells or the use of charcoal stripped-serum growth medium.

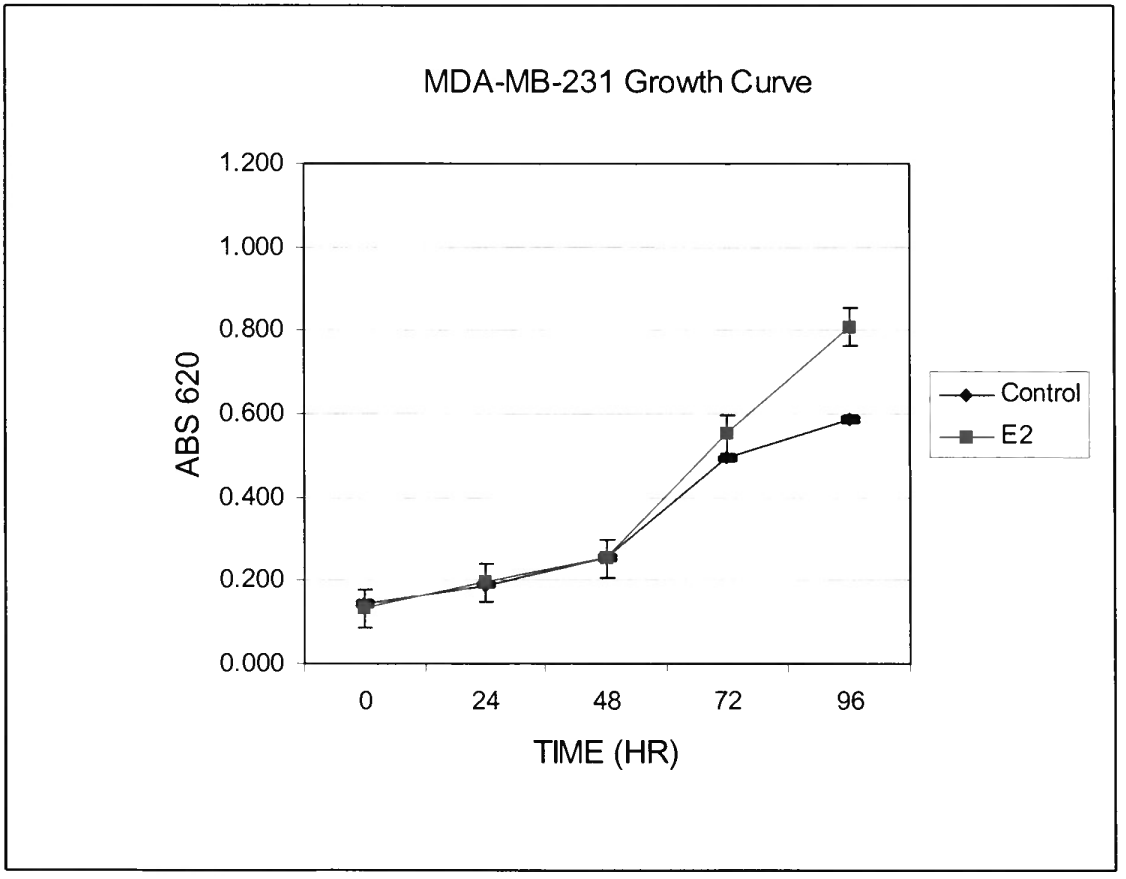


Figure 6. MDA-MB-231 growth curve. MDA-MB-231 cells ($1x10^5$/ml) were treated with E2 and control untreated for 0 – 96 hrs. At 24 hr intervals, viable cells were determined using crystal violet blue stain. The growth curve is an average of three separate experiments performed in duplicate.

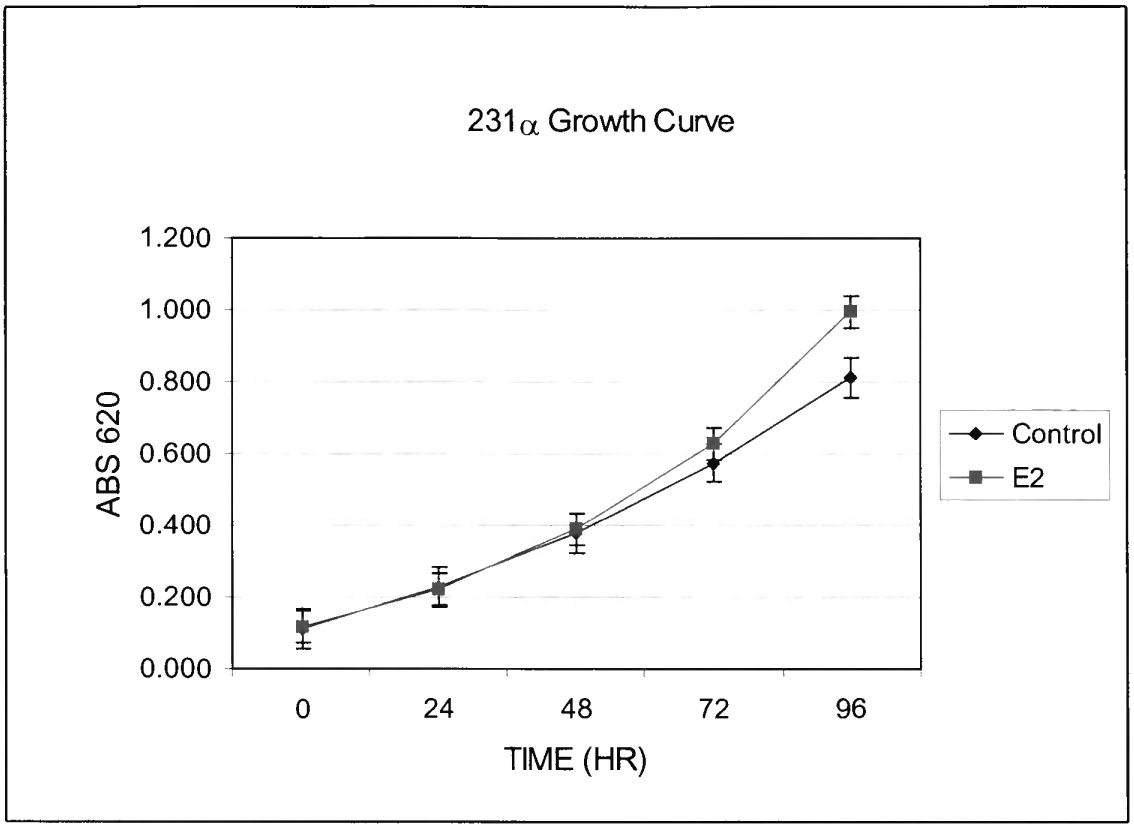


Figure 7. 231α growth curve. 231α cells ($1x10^5$/ml) were treated with E2 and control untreated for 0 – 96 hrs. At 24 h intervals, viable cells were determined using crystal violet blue stain. The growth curve is an average of three separate experiments performed in duplicate.

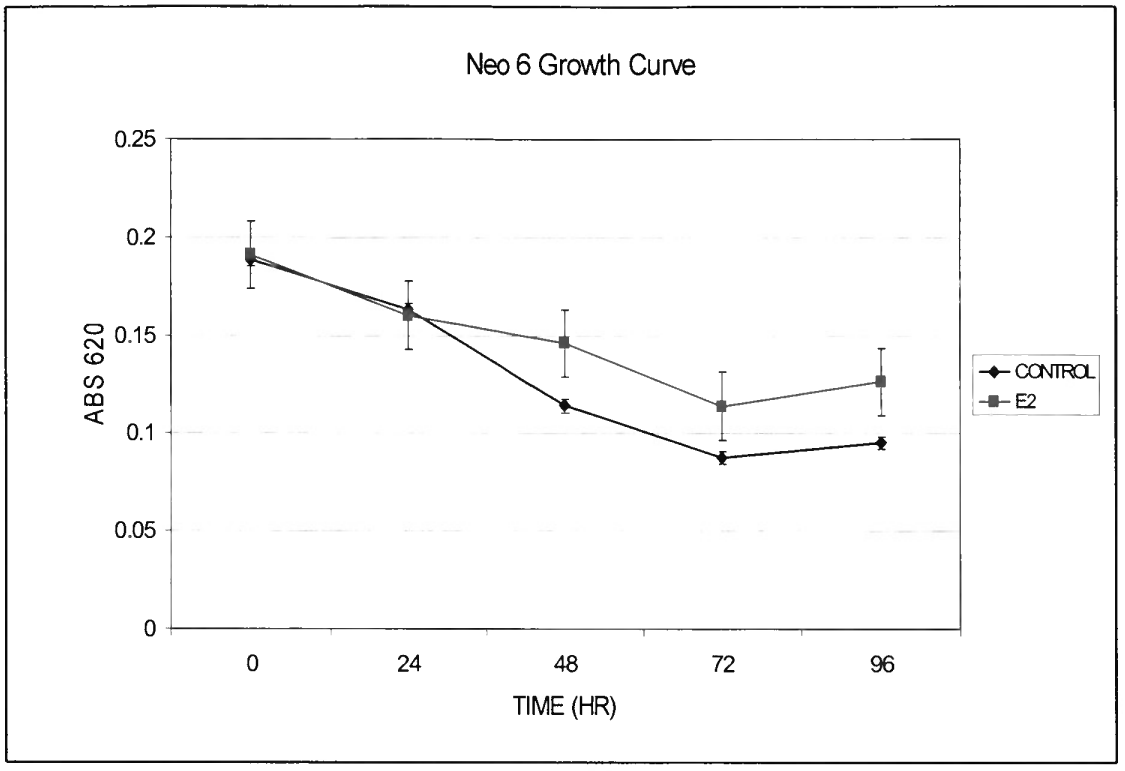


Figure 8. Neo 6 growth curve. Neo 6 cells ($1x10^5$/ml) were treated with E2 and control untreated for 0 – 96 hrs. At 24 hr intervals, viable cells were determined using crystal violet blue stain. The growth curve is an average of three separate experiments performed in duplicate.

4.4 Identification of Estrogen-Regulated Genes

Analysis of microarray data using Affymetrix MAS 5.0 and GeneSpring 6.0 identified over 11,000 genes and express sequence tags (ESTs) that were differentially expressed in 12 hr estrogen treated MDA-MB-231 cells in comparison with non-treated cells. There were 320 genes with a 2-fold or higher increase in expression. Twenty-four of those genes were induced approximately 5-fold or higher (Table 2). There were only 2 genes that were down-regulated 2-fold or less. In 2 hr estrogen treated MDA-MB-231 samples compared with control, there were more than 10,000 genes expressed. Included

in those genes were 209 genes with a 2-fold or higher increase in expression and 9 genes were down-regulated greater than 2-fold. There were 13 genes with a differential expression of 5-fold or higher increase in the 2 hr E2 treated MDA-MB-231 samples (Table 3).

Table 2. Estrogen up-regulated genes at 12 hrs in MDA-MB-231.

Fold Change	Gene Name	Description
18.4	MPDU1	Mannose-P-dolichol utilization defect 1
15.3	STIP1	Stress-induced-phosphoprotein 1
12.1	PFAAP5	Phosphonoformate immuno-associated protein 5
10.9	IL24	Interleukin 24, transcript variant 1
10.6	MAGEA11	Melanoma antigen, family A, 11
10.6	AKR1C2	Aldo-keto reductase family 1 member C2
7.8	RGS4	Regulator of G-protein signaling 4
7	NQO1	NAD(P)H dehydrogenase, quinone1
6.6	KIAA1026	Alpha 1, type 1 collagen preproprotein
6.5	PRO0478	Chromosomal protein
6.5	IL1A	Interleukin 1-alpha
6.4	PSMC3	Proteasome (prosome, macropain) 26S subunit, ATPase 3
6.1	MEST	Mesoderm specific transcript homolog, transcript variant 1
5.8	SMA5	Spinal muscular atrophy 5
5.8	EST: Hs.373565	Transcribed sequence with moderate similarity to Hyp. Protein FLJ11267
5.8	ANGPTL4	Angiopoietin-like 4, transcript variant 2
5.8	C4BPB	Complement component 4-binding protein
5.7	NFYA	Nuclear transcription Factor Y, alpha
5.5	PCDH7	BH-protocadherin (brain-heart), transcript variant a
5.5	C6orf11	Chromosome 6 open reading frame 11
5.5	BUB1	Budding uninhibited by benzimidazoles 1 homolog
5.5	BTF	Bcl-2-associated transcription factor
5.3	PQBP1	Polyglutamine binding protein 1
5.1	DBT	Dihydrolipoamide branched chain transacylase

This table represents genes with a differential expression of >5-fold increase in MDA-MB-231 cells treated with E2 for 12 hr compared with untreated cells.

Table 3. Estrogen up-regulated genes at 2 hrs in MDA-MB-231.

Fold Change	Gene Name	Description
23.2	MPDU1	Mannose-P-dolichol utilization defect 1
12.2	STIP1	Stress-induced-phosphoprotein 1
7.8	RGS4	Homo sapiens regulator of G-protein signaling 4
7.3	DDX9	DEAH box polypeptide 9
7.2	NFYA	Nuclear transcription Factor Y, alpha
6.9	SPTBN1	Homo sapiens spectrin, beta, non-erythrocytic 1
6.2	BTF	Bcl-2-associated transcription factor
6	DDX9	DEAH box polypeptide 9
5.7	BUB1	Budding uninhibited by benzimidazoles 1 homolog
5.6	USP10	Ubiquitin specific protease 10
5.3	MYO1D	EST:nq24b02.s1; Myosin 1D
5.2	CT1	Collagen type 1
5.1	SEC22L1	SEC22 vesicle trafficking protein-like 1

This table represents genes with a differential expression of >5-fold increase in MDA-MB-231 cells treated with E2 for 2 hr compared with untreated cells.

Evaluation of the 231α E2 treated samples revealed that there were over 9,000 genes expressed in 12 hr E2 treated sample compared to control. One hundred twenty-three (123) genes were up-regulated greater than 2-fold; 145 genes were down-regulated 2-fold or more. There were 2 genes that met the 5-fold increase in gene expression cut-off in the 12 hr E2 treated sample compared to control (Table 4) and 5 genes were identified as down-regulated greater than 5-fold in this sample (Table 5). More than 10,000 genes were regulated by E2 in 2 hr 231α treated samples compared to the control. Of these, 108 genes were up-regulated greater than 2-fold and 7 genes were down-

regulated greater than 2-fold. There were 3 genes with 5-fold or greater increase in expression (Table 6), but no genes met the 5-fold decrease in gene expression cut-off in the 2 hr E2 treated 231α sample compared to control.

Table 4. Estrogen up-regulated genes at 12 hrs in 231α.

Fold Change	Gene Name	Description
8.6	IL13RA2	Homo sapiens interleukin 13 receptor, alpha 2
5.5	ZNF492	Zinc finger protein 492

This table represents genes with a differential expression of >5-fold increase in 231α cells treated with E2 for 12 hr compared with untreated cells.

Table 5. Estrogen down-regulated genes at 12 hrs in 231α.

Fold Change	Gene Name	Description
-16.2	PHLDA1	Pleckstrin homology-like domain, family A, member 1
-6.3	LSM4	U6 small nuclear RNA associated protein 4
-5.9	FUS-CHOP	Fusion protein
-5.4	DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)
-5.2	PCIA1	Cross-immune reaction antigen PCIA1

This table represents genes with a differential expression of >5-fold decrease in 231α cells treated with E2 for 12 hr compared with untreated cells.

Table 6. Estrogen up-regulated genes at 2 hrs in 231α.

Fold Change	Gene Name	Description
10.9	IL13RA2	Interleukin 13 receptor, alpha 2
8.2	TIMP3	Tissue inhibitor of metalloproteinase 3
5.1	DDX9	DEAH box polypeptide 9

This table represents genes with a differential expression of >5-fold increase in 231α cells treated with E2 for 2 hr compared with untreated cells.

Comparative analysis of the microarray data from 231α and MDA-MB-231 samples produced lists of genes that were differentially expressed among the cell lines. In the untreated 231α sample compared to the untreated MDA-MB-231 sample, there were over 10,000 genes that were differentially expressed. That included 26 genes that were up-regulated 2-fold or more and 234 genes that were down regulated 2-fold or more. The previously used fold change filter of 5-fold increase or decrease was applied to the data. This revealed 6 genes that were down-regulated 5-fold (Table 7), but there were not any up-regulated genes that met the filter. When data for the 2 hr E2 treated 231α sample was compared to the 2hr E2 treated MDA-MB-231 samples it showed that there were over 11,000 genes differentially expressed. Among the vast number of genes, 212 genes were identified as 2-fold up-regulated and 10 genes were identified as 2-fold down-regulated. Thirteen (13) genes met the 5-fold increase filter (Table 8), but no genes were down-regulated 5-fold. Over 9,500 genes were differentially expressed in the 12 hr E2 treated 231α sample compared to the 12 hr E2 treated MDA-MB-231 sample. One-hundred-twenty five (125) genes were up-regulated 2-fold, while there were 148 genes that were down-regulated 2-fold. The filter identified one (1) gene that was up-regulated (Table 9) and 5 genes that were down-regulated (Table 10).

Table 7. Genes down-regulated at 0 hr in 231α.

Fold Change	Gene Name	Description
-11.4	SLC16A3	Solute carrier family 16 (monocarboxylic acid transporters)
-8.7	LSM4	U6 snRNA-associated Sm-like protein 4
-8.2	PHLDA1	Pleckstrin homology-like domain, family A, member 1
-7.6	DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)
-5.2	H2AFY	H2A histone family, member Y
-5.1	LAMP1	Lysosome-associated membrane glycoprotein

This table represents genes with a differential expression of >5-fold decrease in 231α cells compared with MDA-MB-231 cells.

Table 8. Estrogen up-regulated genes at 2 hrs in 231α compared with MDA-MB-231.

Fold Change	Gene Name	Description
20.6	MPDU1	Mannose-P-dolichol utilization defect 1
19.6	S100P	S100 calcium binding protein P
12.7	STIP1	Stress-induced-phosphoprotein 1
6.9	SPTBN1	Spectrin, beta, non-erythrocytic 1
6	POLD4	Polymerase (DNA-directed), delta 4
5.9	NFYA	Nuclear transcription factor Y, alpha
5.9	PGM5	Phosphoglucomutase 5
5.7	TNPO2	Transportin 2 (importin 3, karyopherin beta 2b)
5.4	TXNIP	Thioredoxin interacting protein
5.1	NNMT	Nicotinamide N-methyltransferase
5.1	DDX9	DEAH box polypeptide 9
5	TRIO	Triple functional domain (PTPRF interacting)
5	PXN	Paxillin beta

The genes in this table were up-regulated >5-fold in 2 hr E2 treated 231α cells compared with 2 hr E2 treated MDA-MB-231 cells.

Table 9. Estrogen up-regulated genes at 12 hrs in 231α compared with MDA-MB-231.

Fold Change	Gene Name	Description
5.5	ZNF492	Zinc finger protein 492

This gene was up-regulated >5-fold in 12 hr E2 treated 231α cells compared with 12 hr E2 treated MDA-MB-231 cells.

Table 10. Estrogen down-regulated genes at 12 hrs in 231α compared with MDA-MB-231.

Fold Change	Gene Name	Description
-16.2	PHLDA1	pleckstrin homology-like domain, family A, member 1
-6.3	LSM4	U6 snRNA-associated Sm-like protein 4
-5.9	FUS1	Fusion gene in myxoid liposarcoma
-5.4	DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)
-5.2	PCIA1	Cross immune reaction antigen PCIA1

This table represents genes with a differential expression of >5-fold decrease in 12 hr E2 treated 231α cells compared with 2 hr E2 treated MDA-MB-231 cells.

Additional analysis using GeneSpring 6.0 (Silicon Genetics, Redwood City, CA) allowed for generating lists of selected genes and for visualizing data by different methods. The similarities and differences between gene expression profiles of two given samples can be visualized using scatterplots. In a scatterplot, each point represents the expression value of a gene in two experiments, one plotted on the X-axis (untreated control) and the other one on the Y-axis (E2-treated) (Figures 9-12). Genes with equal expression values lined up on the central diagonal line, whereas outliers correspond to up- or down-regulated genes. Although the vast majority of genes have transcript levels that remain unchanged, there were outliers in each experimental condition when cells

were treated with E2 at different time points. Most of the outliers were up-regulated genes in E2 treated cells compared with untreated control cells; with the exception being 12 hr E2 treated 231α cells which exhibit more down-regulated genes. This implies that the presence of ERα in an exogenous system causes the repression of select genes.

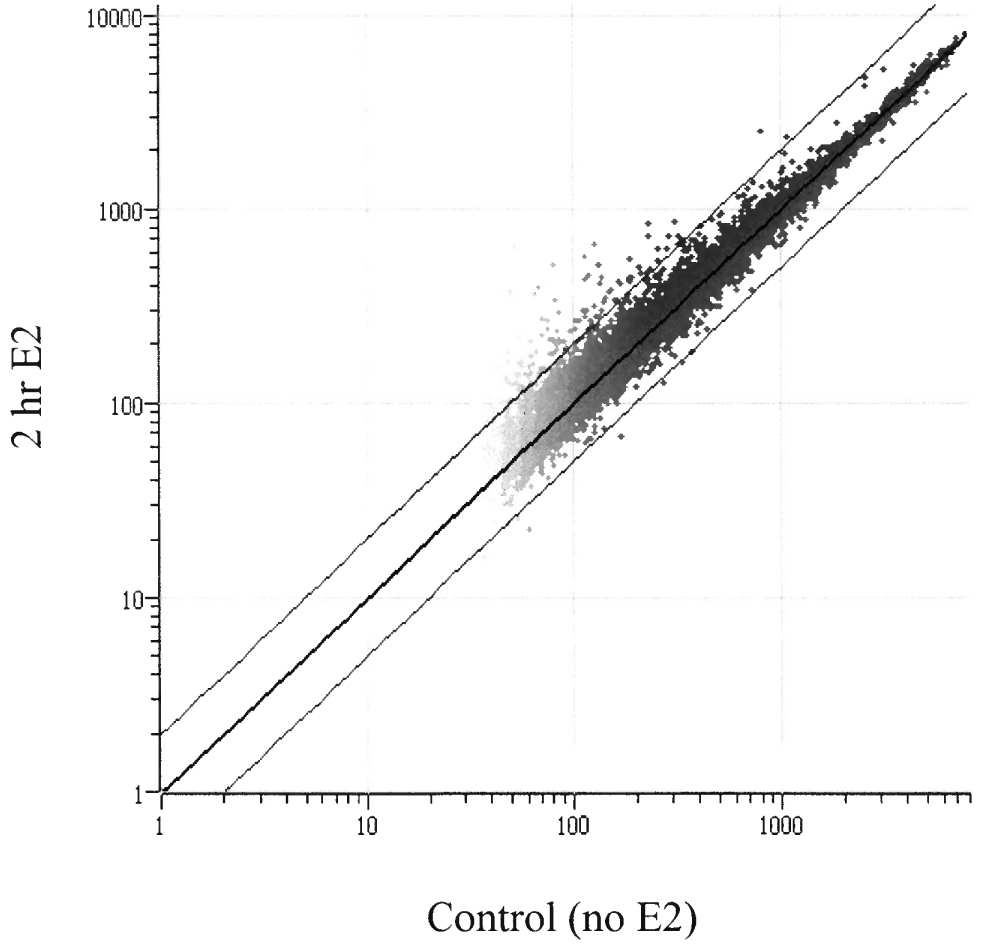


Figure 9. Scatterplot of 2 hr E2 treated MDA-MB-231. Expression profile of the control untreated (X-axis) and 2 hr E2 treated (Y-axis) MDA-MB-231 cells is shown as a bivariate scatterplot of genes from three microarray experiments. The averaged values are normalized adjusted intensities representing levels of expression (scale on each axis).

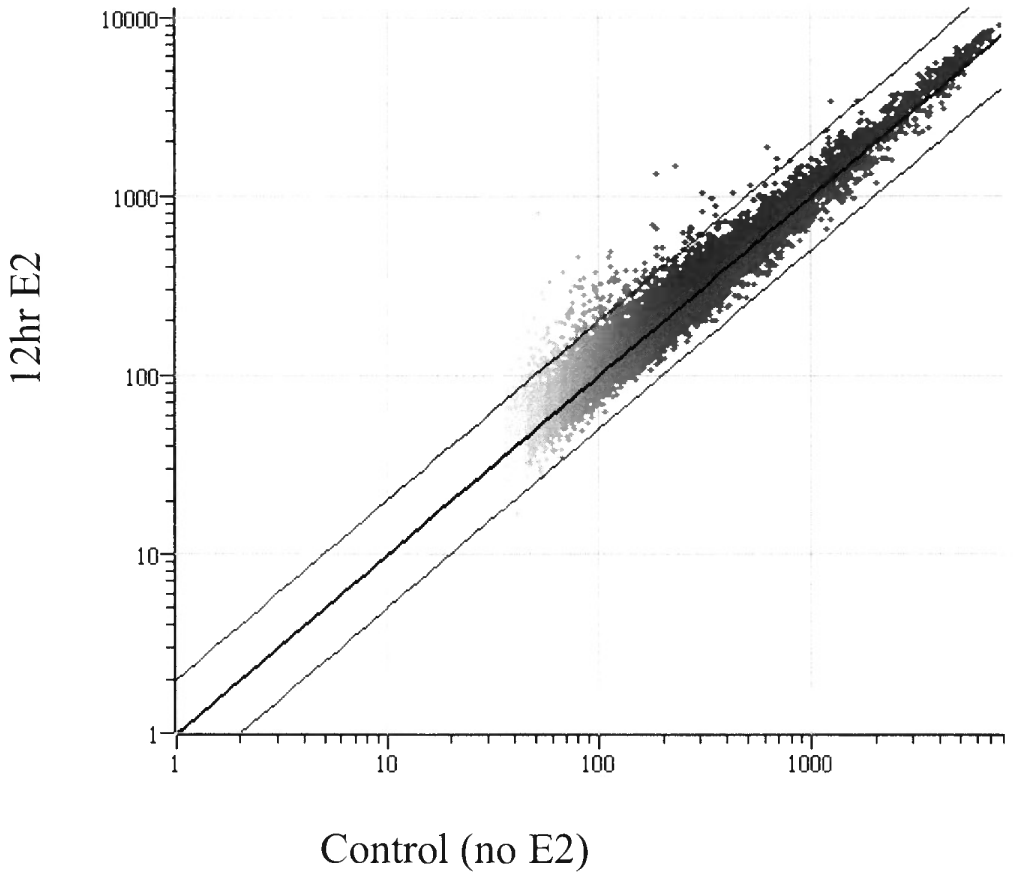


Figure 10. Scatterplot of 12 hr E2 treated MDA-MB-231. Expression profile of the control untreated (X-axis) and 12 hr E2 treated (Y-axis) MDA-MB-231 cells is shown as a bivariate scatterplot of genes from three microarray experiments. The averaged values are normalized adjusted intensities representing levels of expression (scale on each axis).

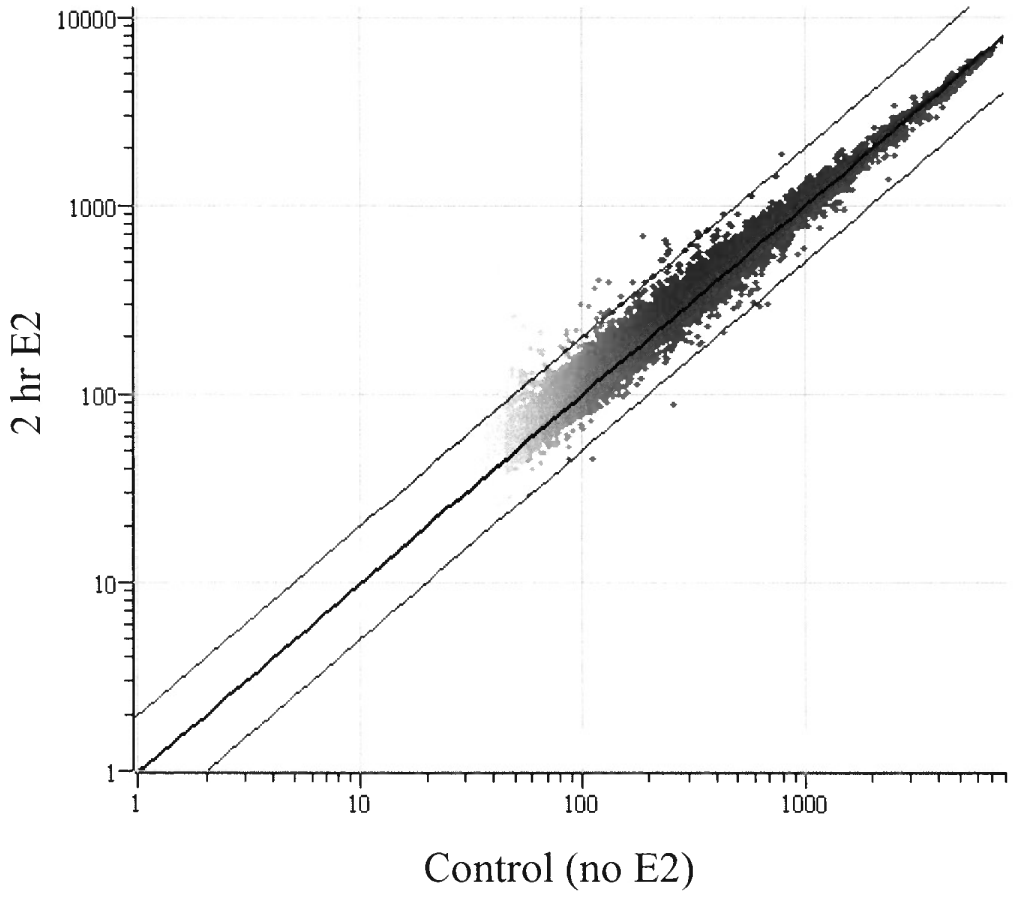


Figure 11. Scatterplot of 2 hr E2 treated 231α. Expression profile of the control untreated (X-axis) and 2 hr E2 treated (Y-axis) 231α cells are shown as a bivariate scatterplot of genes from three microarray experiments. The averaged values are normalized adjusted intensities representing levels of expression (scale on each axis).

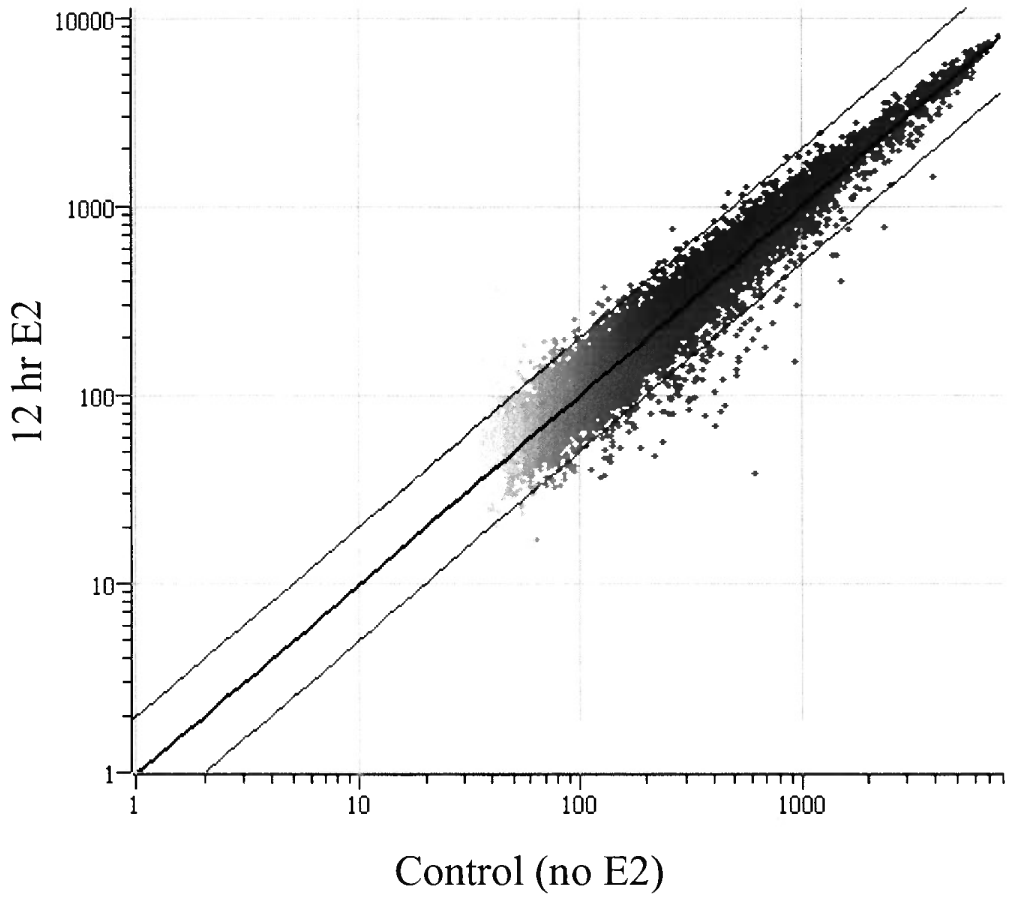


Figure 12. Scatterplot of 12 hr E2 treated 231α. Expression profile of the control untreated (X-axis) and 12 hr E2 treated (Y-axis) 231α cells are shown as a bivariate scatterplot of genes from three microarray experiments. The averaged values are normalized adjusted intensities representing levels of expression (scale on each axis).

Venn diagrams were created to quickly visualize genes common to more than one sample, as well as those present only in a particular sample. The genes illustrated as numbers in the diagrams were extrapolated from three gene lists created for each sample. The three gene lists were subtracted from a pool of more than 22,000 genes in the HG-U133A genome; the number of genes that remain are highlighted in the gray background

above the "all genes" label. Figure 13 and 14 show the number of genes up-regulated 2-fold or higher in the MDA-MB-231 and 231α cells at 2 and 12 hrs, and the number of genes expressed in the control cells (untreated at 0 hr). The genes represented in the diagrams are automatically distributed according to two parameters; time and treatment. In Figure 13, there are 23 genes highlighted in yellow that respond to E2 treatment only. This means the time or length of E2 treatment has no effect on their expression because they were up-regulated in both the 2 hr and 12 hr samples. The 182 genes highlighted in pink represent genes overexpressed at 12 hrs, but are not dependent on the E2 treatment because they are also a part of the untreated (0 hr) gene list. Highlighted in aqua blue, are 128 genes overexpressed at 2 hrs, but not responsive to the E2 treatment because they are members of the untreated (0 hr) gene list. The number highlighted in white, 123, represent the genes that are not dependent on treatment or time because they are members of all three gene lists. The 80 genes highlighted in green represent genes that uniquely respond to the E2 treatment and time at 2 hrs because they are not seen in any other gene list. Likewise, the 142 genes highlighted in red represent genes that uniquely respond to the E2 treatment after 12 hrs only, because they are not part of any other gene list. Highlighted in dark blue are 10,887 genes that were not up-regulated by estrogen in (0 hr) MDA-MB-231 cells. In Figure 14, there are 3 genes highlighted in yellow that respond to E2 treatment only. This means the time or length of E2 treatment has no effect on their expression because they were up-regulated in both the 2 hr and 12 hr samples. The 112 genes highlighted in pink represent genes overexpressed at 12 hrs, but are not dependent on the E2 treatment alone because they are also a part of the untreated (0 hr)

gene list. Highlighted in aqua blue, are 97 genes overexpressed at 2 hrs, but not dependent on the E2 treatment because they are members of the untreated (0 hr) gene list. The number highlighted in white, 11, represents the genes that are not responsive to treatment or time because they are members of all three gene lists. The 209 genes highlighted in green represent genes that uniquely respond to the E2 treatment and time at 2 hrs because they are not seen in any other gene list. Likewise, the 42 genes highlighted in red represent genes that uniquely respond to the E2 treatment after 12 hrs, because they are not part of any other gene list. Highlighted in dark blue are 10,631 genes that are exclusive to the untreated (0 hr) 231α cells. The Venn diagrams illustrate that there are more genes up-regulated by E2 in MDA-MB-231 than in 231α.

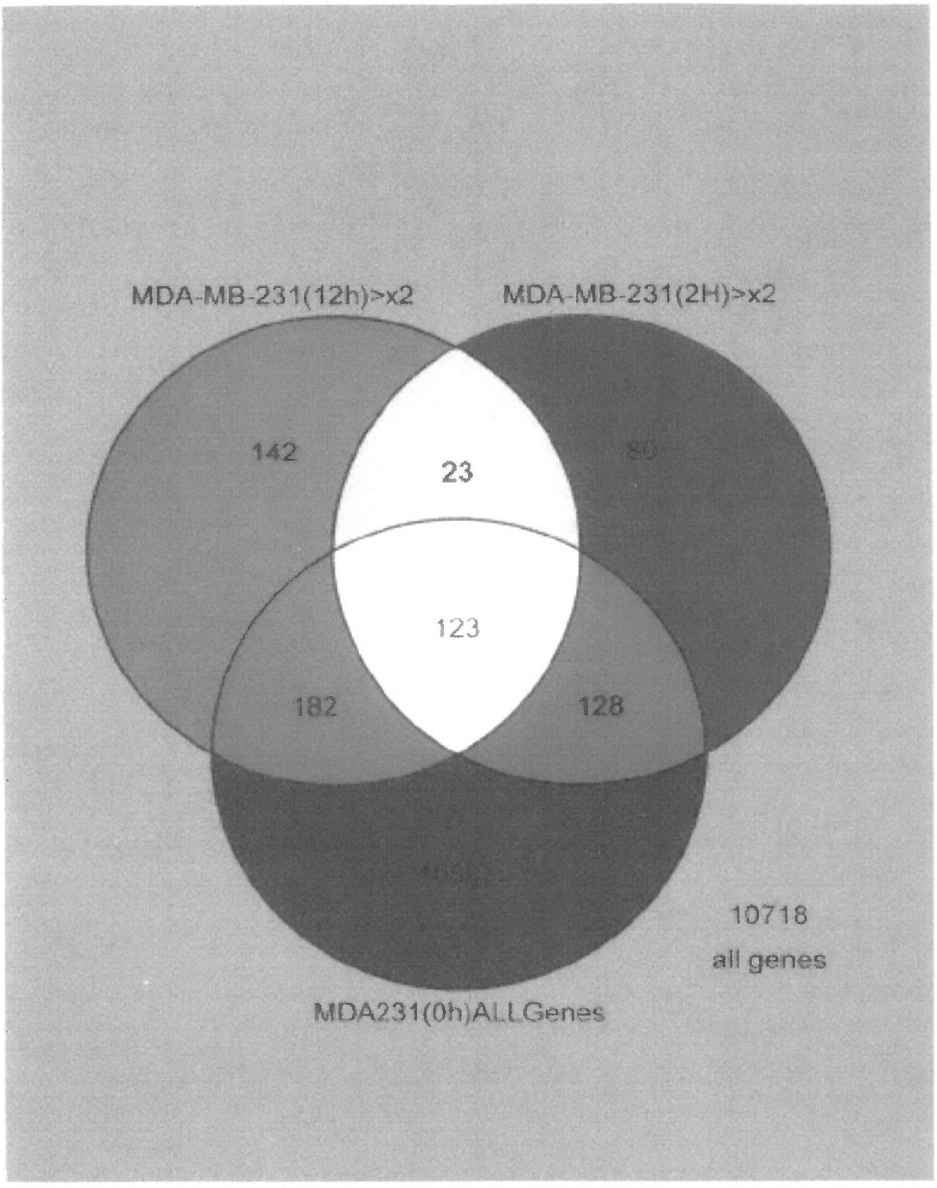


Figure 13. MDA-MB-231 Venn diagram. A Venn diagram was created using genes with a differential expression of 2-fold or more up-regulated in MDA-MB-231 cells treated with E2 for 2 and 12 hrs and all genes expressed in untreated (0 hr) MDA-MB-231 cells.

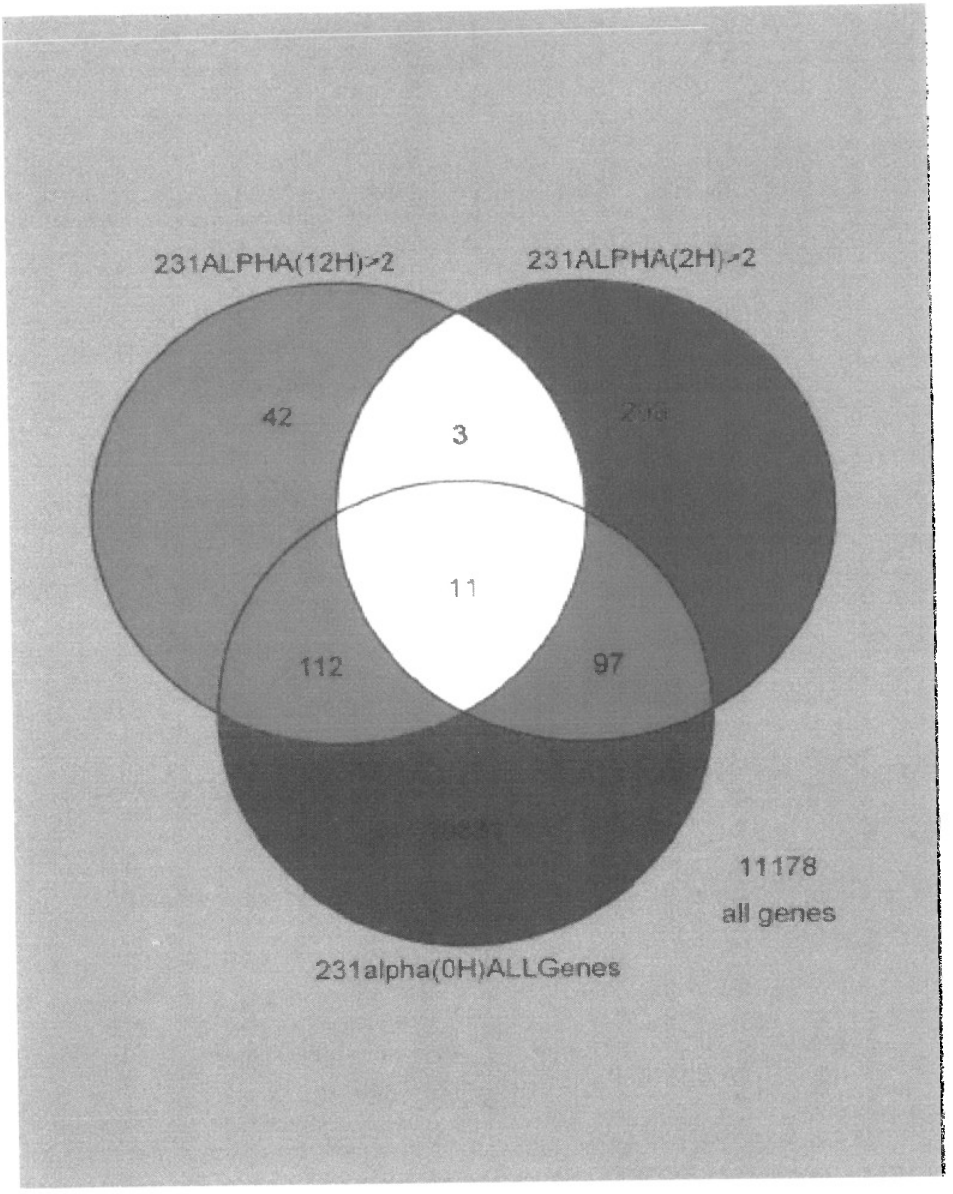


Figure 14. 231α Venn diagram. A Venn diagram was created using genes with a differential expression of 2-fold or more up-regulated in 231α cells treated with E2 for 2 and 12 hrs and genes expressed in untreated (0 hr) 231α cells.

Further analysis of up-regulated genes showed that there were genes significantly up-regulated in treated cells compared with untreated control cells, as well as genes not expressed in the control cells and up-regulated after treatment only. The major functional

categories for the genes (Figures 15 and 16) include apoptosis, cell adhesion, cell cycle, cytoskeleton, enzymes, growth factors/hormones, nucleotide processing, protein processing, receptors, signal transduction, transcription and transporters. Figures 15 and 16 illustrate the number of genes up- and down-regulated by E2 at 2 and 12 hrs in MDA-MB-231 and 231α cells, respectively. As expected, a greater proportion of genes involved in signal transduction and transcription were regulated by E2. These include genes such as signal transducer and activator of transcription 6 (STAT6), insulin-like growth factor 2 receptor (IGF2R), transcription factor 7-like, T-cell specific (TCF7L1), TGF beta-induced factor 2 (TGIF2). It is evident that a greater proportion of genes is regulated by E2 in MDA-MB-231 cells than in 231α cells.

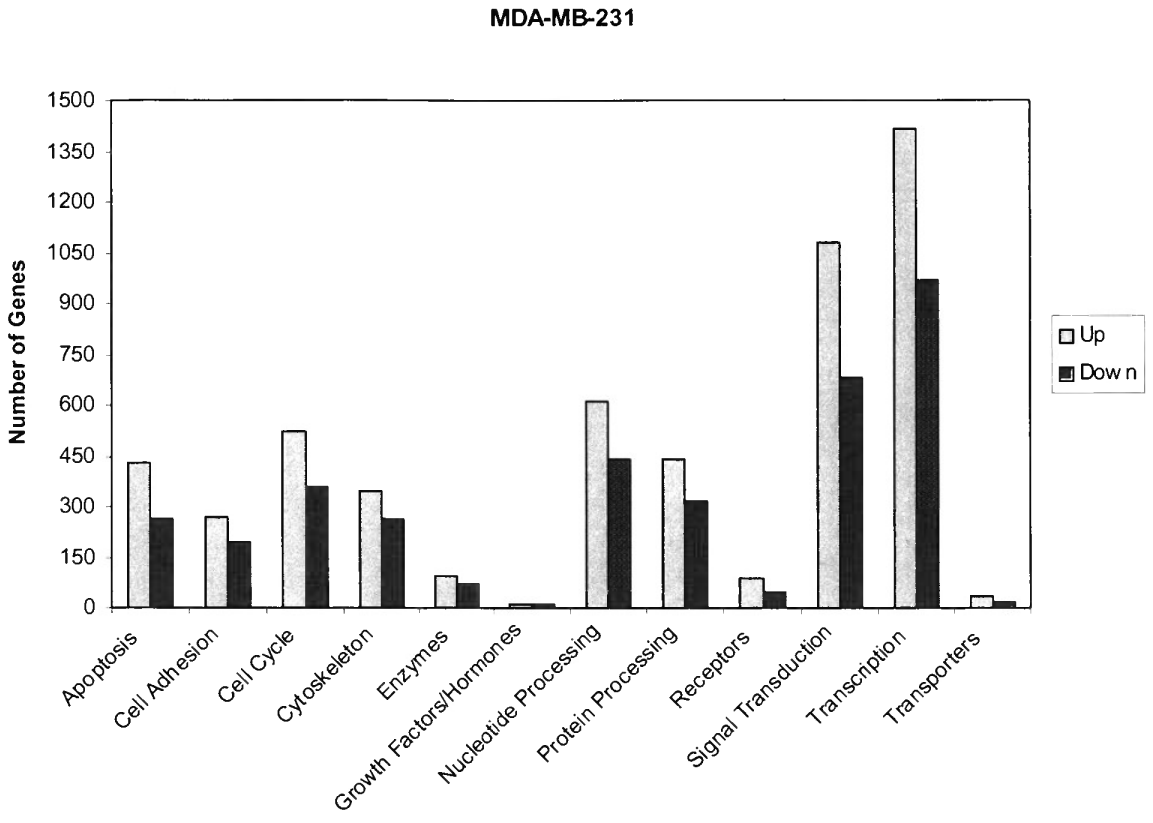


Figure 15. Functional categories of genes regulated by E2 at 2 and 12 hrs in MDA-MB-231 cells. The solid yellow bar represents genes up-regulated by E2 and the solid green bar represents genes down-regulated by E2.

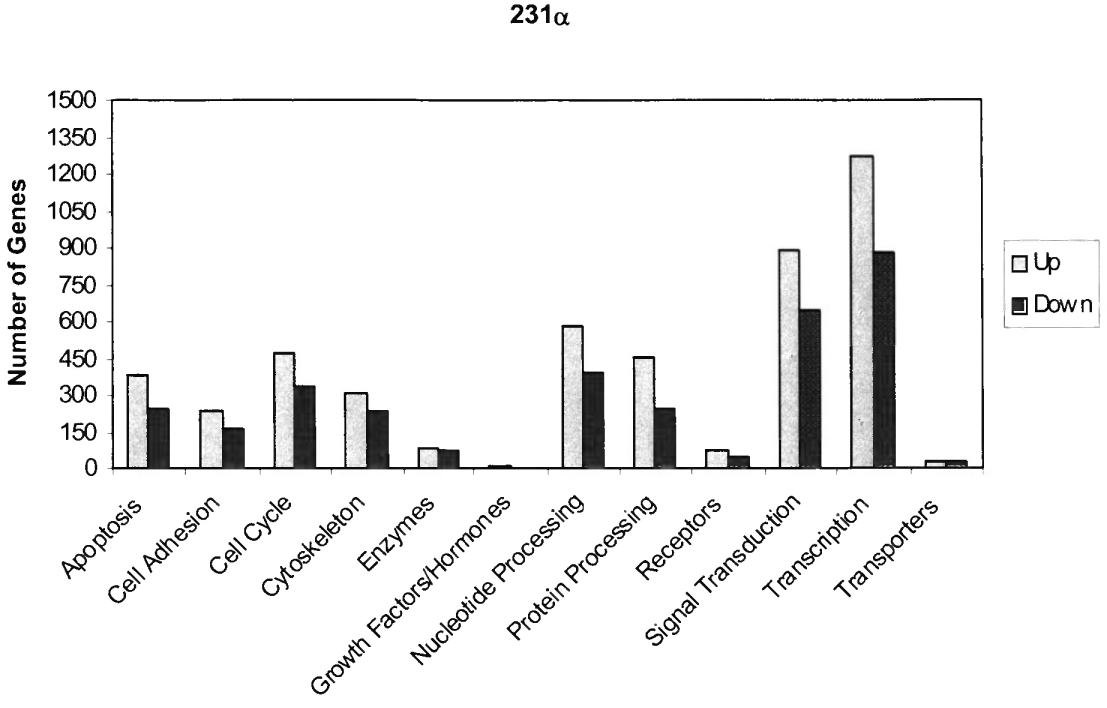


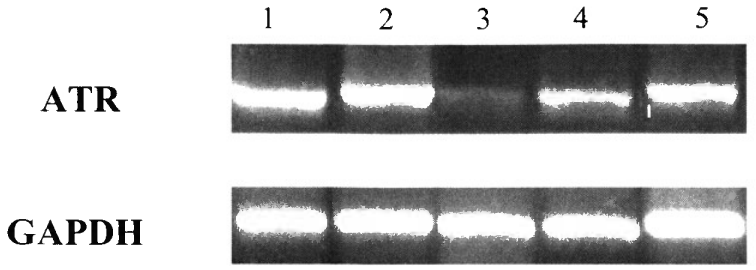
Figure 16. Functional categories of genes regulated by E2 at 2 and 12 hrs in 231α cells. The solid yellow bar represents the genes up-regulated by E2 and the solid green bar represents the genes down-regulated by E2.

4.5 E2 Regulation of ATR

A number of genes associated with the cell cycle are known E2 regulated genes. An example of such are cyclin D1, cyclin A2, cell division cycle 2 (CDC2) and cyclin-dependent kinase inhibitor 1A (Frasor, J. et al. 2003). Evaluation of the genes in the cell cycle category that was created in GeneSpring 6.0, revealed the presence of ATR, ataxia telangiectasia-related protein. Our preliminary data suggested that ATR, also known as FRP1 (FKBP12-rapamycin associated protein (FRAP)-related protein), was induced by E2 at 12 hrs in MDA-MB-231. In 2 of 3 independent microarray experiments, ATR was

up-regulated greater that 5-fold; therefore, the decision was made to analyze this gene further.

Reverse transcriptase PCR was performed to validate the data. Figure 17 shows the regulation of ATR by E2. ATR primers produced a band at an approximate size of 450 bp on a 2% agarose gel in samples of MDA-MB-231 (E2, 2 hrs and 12 hrs) and untreated, MCF-7 and 231α. The relative intensity of the product was higher at 12 hrs and 2 hrs than in the untreated MDA-MB-231 cells, which was lower than the intensity produced in MCF-7 and 231α cells. Primers for GAPDH were also assayed as an internal control. ATR expression was also analyzed in normal (N) and tumor (T) breast tissues (Figure 18). The normal sample, 4N was the only breast tissue sample that did not express ATR. The other samples, which were tumors, displayed various intensities of ATR expression (Figure 18A). These results suggest that ATR plays a role in breast cancer development, but there were not enough normal and tumor breast tissue samples examined to support this claim. We gained access to additional normal and tumor breast tissue samples and screened them for ATR expression (Figure 18B). There were 5 of 6 normal tissues that expressed ATR, while only 3 of 7 tumor tissues expressed ATR. This implies that there is a trend occurring in which ATR is expressed in more normal tissues than tumor tissues.



Lane 1 - MDA -MB -231 (12hr E2)

Lane 2 - MDA -MB -231 (2hr E2)

Lane 3 - MDA -MB -231 (No E2)

Lane 4 - MCF -7 (No E2)

Lane 5 - 231 α (No E2)

Figure 17. E2 regulation of ATR. RT-PCR analysis confirmed the regulation of ATR by E2 with time course treatment of MDA-MB-231 cells. Untreated samples of MCF-7 and 231α cells were assayed for expression of ATR. GAPDH was used as an internal control. (PCR courtesy of collaboration efforts with Dr. Lisa Flowers's laboratory, Emory University School of Medicine.)

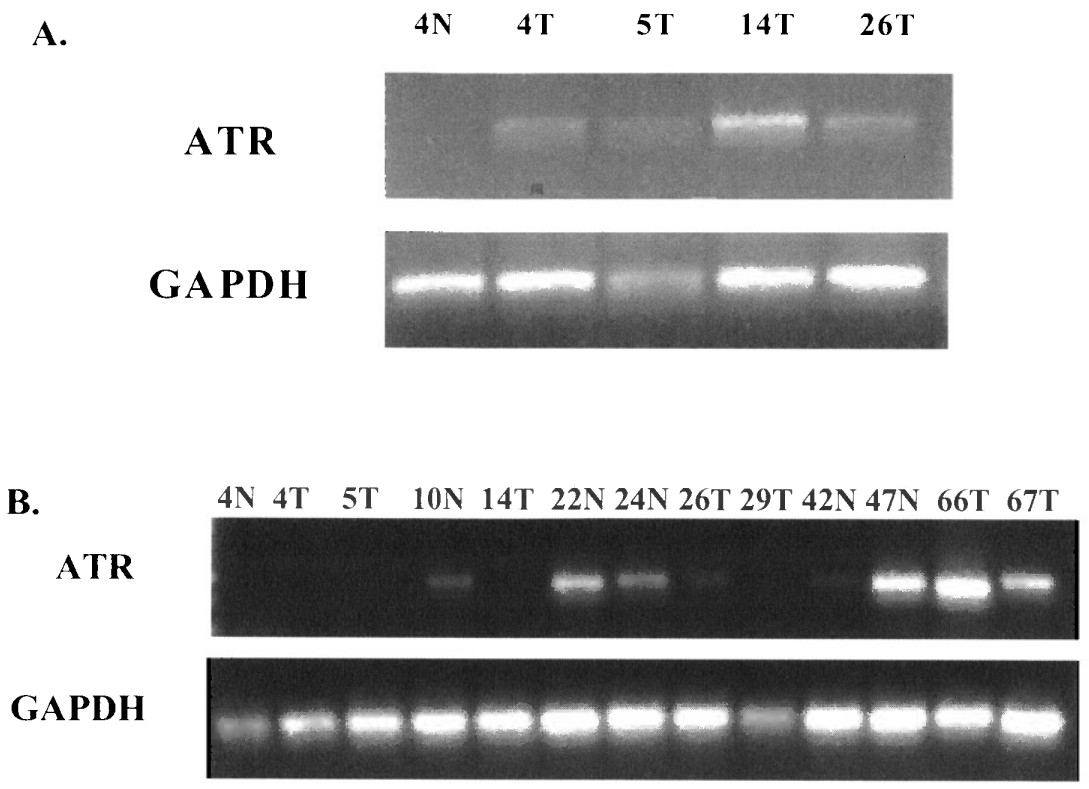
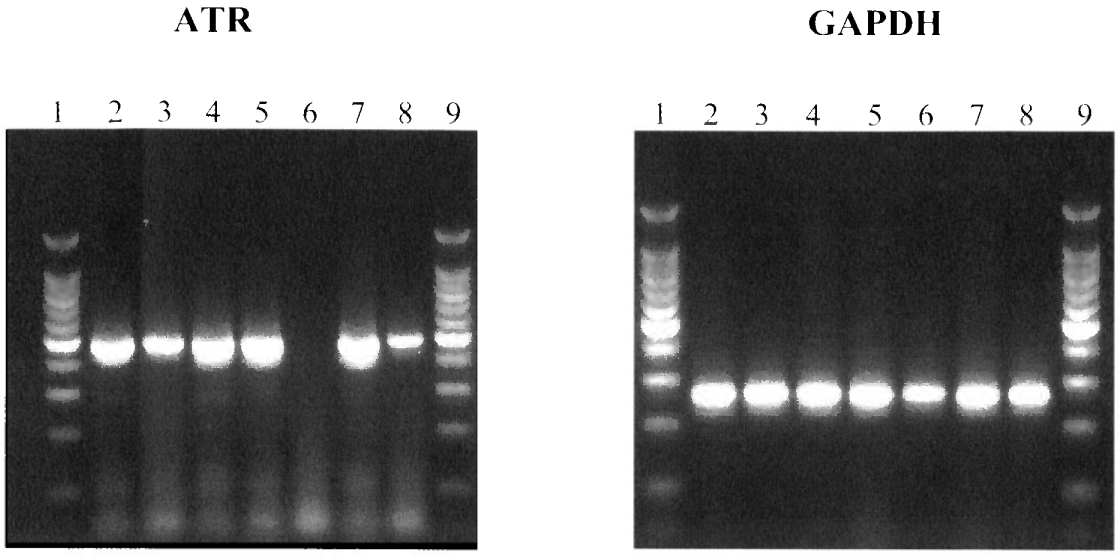


Figure 18. Expression of ATR in breast tissues. RT-PCR analysis was performed using normal (N) and tumor (T) breast tissues to determine ATR expression. (A) Samples N4, T4, T5, T26 are ER+ and T14 is ER-. (B) There are 8 tissues (10N, 14T, 22N, 24N, 26T, 42N, 47N, 66T) that are ER+ and 5 tissues (4N, 4T, 5T, 29T, 67T) that are ER-. GAPDH was used as an internal control. (PCR (A) courtesy of collaboration efforts with Dr. Lisa Flowers's laboratory, Emory University School of Medicine.)

Several other breast cancer cell lines were screened for the expression of ATR. The results are shown in Figure 19. ATR was expressed in all cell lines except the ERα/ERβ negative MDA-MB-435 cell line. The BT 549 breast cancer cell line is also ERα/ERβ negative, but expressed a detectable level of ATR. Therefore, the presence or absence of ERα and ERβ is not associated with ATR expression.



Lane 1 & 9- 100bp marker
Lane 2- MCF-7
Lane 3- MDA-MB-231
Lane 4- Neo 6
Lane 5- 231α
Lane 6- MDA-MB-435
Lane 7- MDA-MB-468
Lane 8- BT 549

Figure 19. ATR expression in various breast cancer cell lines. RT-PCR analysis was used to detect the expression of ATR in other breast cancer cell lines. GAPDH was used as an internal control to confirm approximately equal templates in all samples.

CHAPTER 5

DISCUSSION

Estrogen has an essential role in the development of various tissues and in the maintenance of numerous physiological processes. However, it is also well known that estrogen plays a critical role in the etiology and progression of human breast and gynecological cancers. It was the purpose of this study to determine the effects of estrogen on gene expression of breast cancer cells independent of ERα.

Gene expression profiles from ERα negative and positive breast cancer cells were created and examined following E2 treatment. The breast cancer cell lines utilized in these experiments were MDA-MB-231 and 231α. The MDA-MB-231 cell line is referred to in the literature as being ER-negative because it does not express ERα, but the results provided in this study show that detectable levels of ERβ are expressed (Figure 5). Expression of ERβ and its regulation by E2 have been analyzed in MDA-MB-231 and other ERβ positive breast cancer cells (Vladusic, E. et al. 2000; Liu, S. et al. 2002). The 231α cells were created by stably transfecting ERα into MDA-MB-231 cells. The transfection was successful because the cells expressed ERα (Figure 5). MCF-7 breast cancer cell line was used as a control because it endogenously expresses ERα and ERβ (Liu, S. et al. 2002; Cunliffe, H. et al. 2003). Figure 5 shows faint expression of ERβ in

MCF-7 cells. This could be due to an error in the pipetting of primers or template.

The Neo 6 cell line was created by transfecting the empty pcDNA3 expression vector into the MDA-MB-231 cell line. Success of transfection was determined by screening for the neomycin (NEO) resistant gene, which is incorporated into the vector for selection of stable transfectant cell lines (Figure 4). Neo 6 served as the control cell line for the transfectant cell line, 231α.

The ability of E2 to induce cell proliferation has always been an aspect of interest in E2 studies. It has been well documented that E2 induced cell proliferation of breast cancer cells is relative to the expression of the ERs (Pike, M. et al. 1993). This is in part due to the fact that many of the growth promoting characteristics of estrogen are mediated through its ability to bind the ERs, α and β. In this study, the effects of E2 on the growth of cell lines MDA-MB-231, 231α and Neo 6 were investigated. In the ERα negative MDA-MB-231 cell line, cell growth was slightly increased with E2 treatment after 48 hrs (Figure 6). This effect could be due to the declining levels of E2 over time, which allowed the cells to rebound and re-establish normal growth rate. Overall, E2 did not affect cell proliferation of MDA-MB-231 cells as previously determined by other studies (Levenson, A. and Jordan, V. 1994). E2 had a similar effect on the ERα transfected cell line 231α, but the growth rate was much higher (Figure 7). The cell density doubled every 24 hrs in both control and E2 treated samples, but after 72 hrs the rate increased four times in E2 treated cells. Previous studies by Lazennec et al. (2001) and Jiang et al. (1992) have documented that E2 causes growth inhibition in cells stably

expressing exogenous ERα. There was no inhibitory effect observed in this study. This could be an effect of clonality, in which the exogenous ERα is unable to take on the role of dominant mediator in the presence of endogenous ERβ in our cloned cell line. Studies by Cheng and Malayer (1999) have shown that ERα had no ability to repress or inhibit cell proliferation in the presence of E2; therefore, illustrating that our data is not the first time this has been observed. In Neo 6, there was a substantial decrease in cell proliferation of E2 treated cells as well as in the control cells (Figure 8). It appears that the cells are dying daily instead of exhibiting a normal rate of growth as seen in the previous growth curves. This may be due to another clonal effect resulting from the transfection of the empty vector into the parental cell line, MDA-MB-231. Another aspect to consider is the method utilized to determine cell viability as referenced in the Materials and Methods. The cells are rinsed with dH_2O to remove the crystal violet blue stain. During this process, viable cells may have been washed off causing the appearance of decreased cell proliferation. We have witnessed this before in the laboratory while attempting to determine the effects of E2 on cell proliferation of MCF-7 cells. Taken together, our results further support the past findings by other investigators that MDA-MB-231 cells are non-responsive to estrogen induced proliferation. Furthermore, these results imply that the presence of ERβ does not play a role in proliferation. This probably occurs as a result of ERβ having weaker transcriptional activity in response to E2 compared to ERα (Pettersson, K. et al. 2000).

Microarray technology permits the transcriptional analysis of a large number of genes and gene products simultaneously. Using this technology in the identification of

E2 regulated genes in breast cancer cells is an essential step toward understanding the molecular mechanisms of E2 action. In this study the goal was to identify genes whose expression was regulated by E2 treatment in ERα negative and positive breast cancer cell lines. GeneChip® expression analysis (Affymetrix, Santa Clara, CA) was utilized to study gene expression profiles after E2 activation in MDA-MB-231 and 231α breast cancer cell lines. The results, taken together, illustrated that treatment with E2 altered the expression of a variety of genes; thereby, providing a comprehensive view of the changes induced by E2 on the transcriptional program of human E2-responsive/unresponsive cells. Ultimately, these findings could possibly identify genes that have the potential to serve as diagnostic/prognostic tools for the monitoring of breast cancer.

Data analyses identified several genes that encode for protein kinases that act in DNA recombination, pre-mRNA splicing and signal transduction. There were also genes identified that participated in a variety of biological processes including cell growth, differentiation, pathogenesis, and tumor suppression. These genes can be found in Tables 2-7. A number of genes known to be E2 regulated were found. They include BRCA1-associated protein 2 (BRAP2), vascular endothelial growth factor (VEGF), insulin-like growth factor binding protein (IGFB) and human estrogen receptor-related alpha (hERRa) (Frasor, J. et al. 2003). These findings help validate our data and imply that the ERα negative MDA-MB-231 cell line responds to E2 by mediating activation through alternative pathways. These alternative pathways may include ligand-independent receptor activation, non-nuclear action through cell-surface receptors or oxidative stress-mediated activation. The ER can be activated independently of E2 by growth factors that

increase the activity of protein kinases that phosphorylate different sites on the receptor. Phosphorylation occurs predominantly at specific serine or tyrosine residues and is catalyzed by enzymes such as receptor tyrosine kinases and mitogen-activated protein kinases (Gruber, C. et al. 2002). In the case of the non-nuclear E2 signaling pathway, the rapid events result from direct estrogenic action on cell membranes and are mediated by cell-surface forms of ER. Although these receptors remain mostly uncharacterized, they are thought to resemble their intracellular counterparts (Watson, C. et al. 1999). This activity is linked to the mitogen-activated protein kinase signaling cascade (Kato, S. et al. 1995; Santen, R. et al. 2002; Zhang, Z. et al. 2002). The genotoxic activity of E2 is mediated by the tissue-specific conversion to catechol estrogen (CE) metabolites with subsequent formation of reactive oxygen species (ROS) and unstable CE intermediates (Yager, J. and Liehr, J. 1996; Yager, J. 2000; Liehr, J. 1999; Mobley, J. and Brueggemeier, R. 2004). This process forms free radicals and allows the intermediates to bond covalently to DNA, which validates estrogen's genotoxic activity.

Further inspection of the microarray data was performed with GeneSpring 6.0 software for gene expression data analysis. It allowed the data of two or more given samples to be viewed in order to determine the similarities and differences between gene expression profiles. Scatterplots were created from MDA-MB-231 and 231α samples (Figures 9-12). These images enabled the observation of the pattern of gene expression in E2 treated and untreated samples. It was interesting in this study to find that E2 caused a greater number of genes to be induced in MDA-MB-231 cells and more repressed in

231α cells. These findings are similar to those of Frasor et al. (2003), in which they identified that seventy percent of the genes regulated by E2 in MCF-7 cells were down-regulated. It suggested that some of the estrogen-induced down-regulation of genes could be due to the squelching/sequestering of shared transcriptional coactivators that might be necessary for maintaining basal gene expression (McKay, L. and Cidlowski, J. 1999; Harnish, D. et al. 2000; Speir, E. et al. 2000).

The key observation illustrated in the scatterplots is reinforced by the Venn diagrams (Figures 13 and 14). It is apparent that there are changes in gene expression attributable, not only to treatment, but also to the presence of ERα. The introduction of ERα into the MDA-MB-231 cells caused more genes to be repressed when exposed to E2 than that seen in the wild type cell line. This observation is illustrated further by Tables 8-10. These findings imply that the presence of ERα helps regulate gene expression and assists in confirming that an alternative mechanism for E2 activation may be the reason for the gene expression profile exhibited by MDA-MB-231 cells.

There is an inferred or known function for the proteins that E2 regulated genes encode. Therefore, it was important to sort the genes by functional categories. The number of genes regulated by E2 in each category is illustrated in Figures 15 and 16. These figures further illustrate the difference in the number of genes regulated by E2 between MDA-MB-231 and 231α cell lines. This phenomenon could be due to several reasons. The first issue is that of aneuploidy; whenever foreign cDNA is introduced into an environment that does not express it endogenously there will be changes in chromosome number among the batch of cloned cells. These changes can cause

differences that affect the natural occurrences of the parental cell line. Secondly, endogenous genes may become down-regulated or deactivated with the introduction of exogenous cDNA (Vladusic, E. et al. 2000; Liu, S. et al. 2002; MacGregor-Shafer, J. et al. 2001). Previous works performed in the laboratory of V. Craig Jordan have successfully transfected ERα into MDA-MB-231 cells. Prior to transfection, the MDA-MB-231 cells were cloned and a clone (10A) was selected for transfection studies (Jiang, S.-Y. and Jordan, V.C. 1992). This clone is no longer representative of the wild type MDA-MB-231 cell line because it no longer expresses levels of ERβ (Tonetti, D. et al. 2003). Even though a homogeneous population of cells was created, it changed the phenotype of the parental cells; therefore, compromising any comparisons made between the newly created ERα-positive cell line and the ERα-negative cell line.

An up-regulated cell cycle related gene that was first identified in the preliminary data analysis for 12 hr E2 treated MDA-MB-231 samples was found again by GeneSpring 6.0 analysis of 2 hr and 12 hr E2 treated MDA-MB-231 samples. ATR, ataxia telangiectasia mutated Rad3-related, belongs to the PI3K-related kinase (phosphatidylinositol 3-kinase) family (Cimprich, K. et al. 1996). It has a molecular mass of 301 kDa and functions in signaling the presence of DNA damage, activating cell cycle checkpoints, and repairing DNA. It has also been shown to phosphorylate checkpoint kinase 1, 2 (CHK 1, 2), p53 and breast cancer 1 (BRCA1) (Chen, J. 2000; Bradbury, J. and Jackson, S. 2003). An important effect of these phosphorylation events is the control of cell cycle checkpoints. They ensure that the cell efficiently repairs the DNA damage before DNA replication or cell division occurs.

RT-PCR experiments were performed to confirm that the up-regulation of ATR by microarray experiments was truly estrogen-regulated (Figure 17). The difference in ATR expression was evident in untreated MDA-MB-231 cells compared to E2 treated MDA-MB-231 cells; thereby, validating that E2 induces ATR expression in MDA-MB-231 cells. Our identification of ATR as an E2 responsive gene is the first time this has been shown to date. RT-PCR analysis was performed using normal and tumor breast tissue samples, acquired through collaboration with Dr. Lisa Flowers at Emory University School of Medicine, to screen for ATR expression (Figure 18). ATR was expressed in tumor breast tissue samples, but not in the one normal breast tissue sample assayed (Figure 18A). These results imply that ATR has a role in breast cancer tumors, but an efficient number of normal and tumor breast tissue samples should be examined in order to validate this implication. We screened more breast tissue samples for ATR expression and noticed a trend occurring that implies that ATR is expressed in more normal tissues than in tumor tissues (Figure 18B). This suggests that ATR is adequately correcting the DNA damage that may occur in normal breast tissue. Once again, a larger number of breast tissue samples should be analyzed in order to statistically validate this implication. The expression of ATR was found in various breast cancer cell lines (Figure 19) of different ER status; thereby, suggesting that ER status, in itself, does not determine expression levels of ATR.

Disruption of the mechanisms that regulate cell cycle checkpoints, DNA repair, and apoptosis results in genomic instability and the development of cancer. ATR is one of the central players in checkpoint activation in response to DNA damage, making its

role pivotal in tumor suppression (Motoyama, N. and Naka, K. 2004). The identification of ATR as an E2 induced gene in MDA-MB-231 breast cancer cells providesimplications for its use in cancer therapy. Currently, there are studies being conducted that emphasize checkpoint kinases and their potential as cancer drug targets (Zhou, B. et al. 2003). Studying the signaling events that occur when ATR responds to DNA damage would identify the proteins most commonly responsible for inadequate DNA repair and tumor suppression. Therapeutic drugs could then be designed to target those specific proteins in order to suppress genomic instability, which is an important cause of tumorigenesis. Investigations by Yamane et al. (2004) have indicated that the ATR-Chk1 pathway is involved in G2/M checkpoint response to DNA mismatches. This is important because DNA mismatch repair deficiency in cancers is associated with resistance to a myriad of clinically active chemotherapy drugs, thereby, providing insight into the reason many ERα negative breast tumors develop drug resistance. If the mechanism(s) responsible for preventing DNA mismatch repair is determined, it could lead to the development of drugs that promote cancer treatment instead of enabling cancers to exhibit resistance to treatment.

ATR expression results as a response to DNA damage (Tibbetts, R. et al. 2000; Zhou, B. and Elledge, S. 2000). The form of DNA damage must be specific, such as bulky lesions or stalled replications (O'Connell, M. et al. 2000; Khanna, K. et al. 2001; Shiloh, Y. 2001; Foray, N. et al. 2003). As seen in this study, E2 induces ATR expression in breast cancer cells through ER independent mechanisms. This leads to the

assumption that E2 is causing additional DNA damage, perhaps through an oxidative stress-mediated pathway.

There are many reports that establish a link between estrogen-induced breast cancer and oxidative stress (Cavalieri, E., et al. 2000; Yager, J. 2000, Yager, J. et al. 1996; Chen, Y. et al. 2000; Jefcoate, C. et al. 2000). A variety of estrogens are capable of acting as complete carcinogens through a mechanism that involves oxidative stress in the kidney, liver, and breast tissues of various rodent models (Mobley, J. and Brueggemeier, R. 2004; Han, X. and Liehr, J. 1994). Therefore, the possibility exists that E2 treatment of MDA-MB-231 cells can cause the formation of reactive oxygen species (ROS), which initiate DNA damage that activate ATR. This phenomenon can be studied by treating MDA-MB-231 with E2, while measuring the changes in antioxidant status and sensitivity to DNA damage by peroxide. Estrogen-mediated oxidative DNA damage in mammary gland epithelia has been found to induce 8-oxo-2'-deoxyguanosine, thereby, suggesting a role for oxidative stress in the initiation and/or progression of breast neoplasia (Tagesson, S. et al. 1995; Wani, G. et al. 1998; Mobley, J. and Brueggemeier, R. 2004). It has been reported that physiologic concentrations of E2 can cause a decrease in catalase activity followed by an increase in glutathione peroxidase (GPx) activity in cultured normal human breast epithelial cells (Dabrosin, C. et al. 1998). There has also been reported changes in the antioxidant profile of breast cancer patients; including decreased catalase activity, decreased glutathione levels in sera, and changes in superoxide dismutase, GPx and glucose-6-phosphate dehydrogenase activities in tissues (Afrasyap, L. et al. 1998; Li, J. et al. 1998). These observations suggest that oxidative

stress induced DNA damage can occur from E2 exposure. This can be addressed by a series of experiments; including measuring estrogen-induced changes sensitive to peroxide-induced DNA damage, peroxide metabolism, total glutathione levels and enzyme activity assays (for example catalase and GPx activities) and western analysis to confirm enzyme studies.

The expression of thousands of genes can now be determined simultaneously using microarray analysis. This study presented analysis of the observed patterns of gene expression in MDA-MB-231 and 231α breast cancer cells untreated and treated for 2 and 12 hrs with E2. There have been several studies of gene expression analysis of breast cancer cells treated with estrogens and selective estrogen receptor modulators relative to the activation of ERα, producing profiles of responsive genes. Even though these studies are valuable, there are only a few studies that address the need for developing catalogues of E2 responsive genes expressed in an ERα negative system. The data address this issue and provide a number of additional opportunities for future studies. Many of the regulated genes, including some very responsive genes, are ESTs. Investigation of these genes is likely to identify biochemical pathways relevant to the growth and differentiation of breast cancer cells that are not ERα positive. The power of gene clustering to define both distinct and overlapping patterns of gene expression associated with E2 can be demonstrated when all experimental conditions are analyzed together. Hierarchical clustering of this data would identify genes relevant to breast cancer in hormone independent cells. These expression clusters would allow the identification of distinct biological pathways regulated by E2 and provide the basis for future mechanistic studies.

In this study, we were able to identify genes regulated by E2 independent of the ERs in breast cancer cells. We also showed that the presence and/or absence of ERα affect the expression pattern of genes regulated by E2. A novel E2 regulated gene (ATR) associated with the cell cycle was identified and helped elucidate a possible alternative pathway for E2 activation. Ultimately, this study helps to provide a better understanding of estrogen's influences on breast cancer and offers improved therapeutics for individuals with breast tumors that are unresponsive to hormone therapy and those that develop hormonal drug resistance.

CHAPTER 6

CONCLUSION

The purpose of this project was to identify genes that were regulated by estrogen in an ERα-negative and positive breast cancer cell line. Differential microarray technology was utilized to study gene expression profiles after estrogen activation in MDA-MB-231 and 231α human breast cancer cells. The results identified a diversity of genes whose expression was altered by estrogen. There were known estrogen-responsive genes that were expressed. These included VEGF, IGFB, BRCA2 and hERRa. In addition, a cell cycle associated gene was found to be up-regulated by estrogen that has not been shown to be estrogen-responsive to date. The gene is referred to as ATR and is a member of a family of PI3K-related protein kinases. Preliminary results showed that it was up-regulated 6.8-fold in 12 hr estrogen treated cells. RT-PCR data confirmed that ATR was truly estrogen induced. Our data suggest ATR expression may not be associated with expression of ERs in breast cancer cell lines and it may occur more often in normal breast tissue than tumor breast tissue. Based on these results, we concluded that the induction of ATR expression by estrogen was mediated through non-ER mechanisms.

In conclusion, this study has addressed the need for developing catalogues or profiles of genes regulated by E2 in an ERα-negative system, indicating that there are

non-ERα mechanisms mediating E2 activation. These findings will provide insight for effective treatment of breast cancers that are unresponsive to hormonal therapy, as well as those that develop drug resistance. The identification of estrogen-regulated genes in breast cancer cells is an essential step toward understanding the molecular mechanisms of estrogen action.

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